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Characterization of a Microbial Community in Response to MTBE Contamination in Marine Sediment

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CHARACTERIZATION OF A MICROBIAL COMMUNITY IN RESPONSE TO MTBE CONTAMINATION IN MARINE SEDIMENT

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by
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ABSTRACT

On March 9, 2015, the *Carla Maersk*, which was carrying 216,049 barrels of methyl tertiary-butyl ether (MTBE) outbound for Venezuela, was struck port side by the bow of the *Conti Peridot.* An unspecified amount of MTBE spilled into the Port of Houston. MTBE has an exceptionally high water solubility and a large volume of the contaminant likely diluted and dispersed throughout the channel. MTBE was the most common fuel oxygenate because of its low cost, ease of production, and favorable transfer and blending characteristics. After only a few years of intense use, MTBE has become one of the most frequently detected groundwater pollutants in the United States. A potential fuel oxygenate alternative is tert-butyl alcohol (TBA). TBA can be found with MTBE in gasoline as a manufacturing by-product. It is also a key intermediate in the degradation of several dialkyl ethers used as fuel oxygenates, including MTBE, which is formed via cleavage of the ether bond. MTBE and TBA are difficult to remediate based on chemical and physical properties and need special consideration in site characterization and remedial design. Some treatment technologies that have been widely used for MTBE and TBA groundwater remediation includes groundwater pump and treat, air sparging, in situ chemical oxidation, phytoremediation, natural attenuation and bioremediation.

Marine sediment samples were collected from the Port of Houston at four different locations. Two locations were approximately the same geographical location as Morgan's Point where the spill occurred, and two additional locations were selected

further downgradient of the shipping channel where MTBE was likely to travel due to boat traffic. In addition, six different locations from the Gulf of Mexico were provided by Battelle for background comparison. These six locations from Battelle will provide data on the typical composition of marine microbial communities and will allow for comparison to microbial communities that had been exposed to MTBE for several months. To the possibility of native bioremediation, each site would be amended with a different terminal electron acceptor and either MTBE or TBA as the amended electron donor. To characterize how the microbial community responds to MTBE contamination in marine sediment, and to identify specific microbial processes that will attenuate MTBE, DNA was extracted from all samples, PCR amplified, and sequenced using Illumina MiSeq high throughput sequencing technology. The samples are prepared for sequencing but the results are for future work.

Looking at the Houston MTBE control for the four sites, it can be seen that each concentration after 55 days were either equal or greater than 100%. This indicates that no chemical reactions resulting in non-biological degradation occurred. All four sites for the Houston MTBE and Fe(III) and Houston TBA and Fe(III) amended bottles remain at a concentration near 80%, although each bottle has a steady linearly decreasing concentration over the 55 days. Houston MTBE and nitrate bottles showed mixed results. Sites two and four remain at approximately 100% of the initial concentration while sites one and three both degraded at nearly the same rate to a final concentration of 79% and 76%, respectively, of initial concentrations. Overall, site three seemed to show the most potential for degradation of the four Houston sites. Because only one bottle was used

instead of duplicates or triplicates in this study, it could be due to the selective nature of the sampling. In other words, site three may have had a higher population of MTBE degrading microorganisms then the other sites. Additionally, given the physical and chemical properties of MTBE, it is possible that MTBE spilled at site four traveled with water quickly before settling at site three.

DEDICATION

This work is dedicated to my loving family and friends. Without their support I would have not gotten to where I am today. Thank you.

ACKNOWLEDGMENTS

I'd like to personally thank my advisor, Dr. Kevin Finneran. His knowledge and guidance allowed me to achieve a level of education few have reached. Thank you to my committee members Dr. David Ladner and Dr. Cindy Lee. They have both helped paved the way for my development academically, professionally, and personally. Thank you to National Science Foundation for the RAPID Response Grant that made this research possible. Thank you to Environmental Research and Education Foundation for electing me as a scholar for 2015. Your belief in me drove me to success.

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INTRODUCTION

On March 9, 2015, at about 1231 central daylight time, the 623 foot long bulk carrier *Conti Peridot* collided with the 599 foot long chemical tanker *Carla Maersk* in the Port of Houston shipping lane near Morgan's Point. The exact location of Morgan's Point can be seen in Figure 1. The *Carla Maersk*, which was carrying 216,049 barrels of methyl tertiary-butyl ether (MTBE) outbound for Venezuela, was struck port side by the bow of the *Conti Peridot.* The collision ruptured the two port wing ballast tanks and the number 4 port cargo tank spilling an unspecified amount of MTBE into the Port of Houston. The United States Coast Guard classified the accident as a major marine casualty, closing one of the nation's busiest ports for three days (National Transportation Safety Board, 2015). To limit any health hazard, a high-density foam was used to trap flammable vapors from the ruptured tanks on the ship due to the volatile nature of MTBE in open atmospheric containers (Agency for Toxic Substances & Disease Registry, 2015). MTBE has an exceptionally high water solubility and a large volume of the contaminant was likely diluted and dispersed throughout the channel. The typical surface water response for spills of this magnitude includes putting hydrophobic skimming booms to contain the contaminants; however, given the hydrophilic nature of MTBE, this response did not contain the chemical. MTBE that reaches marine sediment may persist given its relative recalcitrance. This research provides Port of Houston stakeholders with data to assist on how to best respond to not only this spill, but also future high concentration releases in short and long term response.

BACKGROUND

Fuel oxygenates are used heavily as gasoline additives to improve combustion efficiency and enhance the octane of gasoline. The aim of fuel oxygenates is to reduce the emissions of airborne toxics, carbon monoxide and volatile organic compounds (Wei and Finneran, 2009). Methyl tert-butyl ether (MTBE) was the most common fuel oxygenate because of its low cost, ease of production, and favorable transfer and blending characteristics. MTBE can be produced at refineries with existing technology already in place, blends thoroughly without separating from gasoline, and can be transferred through existing pipelines (Squillace et al. 1997). MTBE was first used in the United States (US) in the 1970s but its use increased tremendously after the 1990 Clean Air Act Amendments that established tighter pollution standards for emissions from automobiles and trucks. MTBE production in the United States peaked in 1999 at over 9200 million kg $year^{-1}$ (Youngster, 2010; Haggblom et al. 2007). In August 2005, the US Energy Bill was passed which removed the oxygenate mandate. As of February 2006, MTBE was no longer used in gasoline for US consumption for liability purposes and was most often replaced by ethanol (Impacts of the Energy Policy Act of 2005*,* n.d.). MTBE produced in the US is used only for export and chemical end-uses (*ICB* Chemical Profile, 2009).

After only a few years of intense use, MTBE became one of the most frequently detected groundwater pollutants in the United States. Inadequate fuel storage systems led to more than 400,000 leaking underground storage tank sites in the United States identified by the US Environmental Protection Agency (EPA) and similar findings are emerging in Europe (Schmidt et al. 2004). The estimated cost of MTBE cleanup from

United States public water supplies ranges from $4-85 billion (AWWA, 2005). Other pathways of MTBE contamination in water resources include leaked pipelines, storm runoff, and precipitation. There have been reports of MTBE contamination in lakes and coastal environments as a result of motorized watercrafts (2002NJ6B, 2002).

A potential fuel oxygenate alternative is tert-butyl alcohol (TBA). TBA can be found with MTBE in gasoline as a manufacturing by-product. It is also a key intermediate in the degradation of several dialkyl ethers used as fuel oxygenates, including MTBE, which is formed via cleavage of the ether bond (ITRC, 2005). Unlike MTBE, TBA is widely used as a solvent and intermediate in industrial processes. While MTBE is considered a potential human carcinogen and has been associated with reproductive mutations in aquatic life, TBA is a known animal carcinogen raising concerns over contaminated sites (Schmidt et al. 2004; Moreels et al., 2006). There are no federal drinking water standards or maximum contaminant levels (MCLs) for MTBE and TBA, although the EPA has set a MTBE advisory level for drinking water at 20-40 μg/L because of the unpleasant odor and taste it creates even at low concentrations (US EPA, 1997). This leaves states to establish their own standards, resulting in a wide range of standards across all states. (ITRC, 2005).

MTBE and TBA are difficult to remediate based on chemical and physical properties. MTBE has a water solubility of approximately 50 g/L and TBA is miscible with water. Neither compound adsorbs well to subsurface solids due to their low octanol-water partitioning coefficients (Log Kow = 1.24 for MTBE and Log Kw = 0.35 for TBA), although some subsurface adsorption will occur to soils, deeper materials in the

unsaturated zone, and aquifer materials. These parameters are problematic because the high solubility and low sorption result in high concentrations of contaminants in surface and ground water, especially when contaminated by a point source. Under most circumstances these contaminants move at velocities similar to the velocities of groundwater and are often found at the leading edge of the plume (Squillace et al. 1997; ITRC, 2005). The fuel water partition coefficient, K_{fw}, is 16 and 0.24 for MTBE and TBA, respectively. A K_{fw} 70 times lower for TBA than for MTBE indicates that there is more efficient transfer of TBA from a non-aqueous phase liquid (NAPL) to water (Schmidt et al. 2004; Zwank et al., 2002). The dimensionless Henry's Law constant is a partitioning coefficient for the gas and aqueous phases. MTBE and TBA have dimensionless Henry's Law constants of 0.024 and 5.72E-04, respectively, at a temperature of 25°C. Values of 0.05 or larger lead to volatilization from water (ITRC, 2005). The low values for MTBE and TBA are the reason why fuel oxygenates partition substantially into water and why these contaminants are hard to remove from water through remediation strategies such as air stripping (Schmidt et al. 2004; ITRC, 2005).

The physical properties of MTBE and TBA are much different than the gasoline components with which they are most frequently found. They need special consideration during site characterization and remedial design. There are many other factors that need to be considered when selecting an appropriate remediation strategy. Some of these factors include ability to meet treatment goals, ability to comply with federal, state, and local requirements, cost, time, community acceptance, reliability, and commercial availability. Some treatment technologies that have been widely used for MTBE and

TBA groundwater remediation includes groundwater pump and treat, air sparging, in situ chemical oxidation, phytoremediation, and natural attenuation (ITRC, 2005).

One treatment technology that has consistently gained attention as a viable option is bioremediation. Bioremediation involves the growth of certain microorganisms that use the contaminants as a source of food and energy. Microorganisms take in nutrients and contaminants and reduce them to less harmful products while gaining energy and carbon needed for cell maintenance and reproduction (US EPA, 2012). Bioremediation effectiveness is site specific and depends on a number of factors such as temperature, pH, redox potential, availability of nutrients and water, and contaminant concentration and composition among others. Some advantages to using a bioremediation strategy include the possibility to completely break down contaminants into other nontoxic chemicals without transferring them into another phase, the low cost of treatment per unit volume, minimal and low-technology equipment is required, it can be implemented in-situ or ex-situ depending on conditions, and it is generally perceived positively by the public because it is a natural process (Sharma & Reddy, 2004; Vivaldi, 2001).

Bioremediation of MTBE and TBA can occur under both aerobic and anaerobic conditions. Aerobic degradation of MTBE and TBA through bioremediation has been studied in depth for both surface water sediment and aquifer sediment (Bradley et al., 2001a; Bradley et al., 2001c; Bradley et al., 1999; Kane et al., 2001; Schirmer et al., 2003). Microorganisms capable of degrading MTBE and/or TBA aerobically have been isolated or studied as part of MTBE-degrading consortia (Deeb et al, 2000; Hanson et al., 1999; Mo et al., 1997; Munoz-Castellanos et al., 2006; Zhong et al., 2007; Raynal and

Pruden, 2008; Salanitro et al., 1994). The common aerobic classes include *Bacillus,* *Rhodococcus,* and *Micrococcus* (Hao et al., 2012). These cultures degrade MTBE with oxygen as the terminal electron acceptor and require dissolved oxygen concentrations greater than 2 mg/L (Wei and Finneran, 2009).

Anaerobic MTBE and TBA biodegradation has been reported for most environmentally relevant terminal electron acceptors (excluding TBA biodegradation under methanogenic conditions), but results are controversial and contradictory in the literature (Schmidt et al., 2004). MTBE and TBA contamination in the subsurface soils and groundwater is almost always under anaerobic conditions because rapid depletion of oxygen results from the metabolism of other, more easily degraded components of gasoline (preferential carbon sources). Therefore, bioremediation by anaerobic microorganisms is an important avenue to explore for the remediation of MTBE and TBA (Sun et al., 2012; Finneran and Lovely, 2001). The common anaerobic classes' capable of MTBE degradation that have been identified include *Proteobacteria,* *Pseudomonas,* and *Thermotogae* (Hao et al., 2012). For anaerobic biodegradation to be used as a reliable method of remediation, more detailed information about the microorganisms involved and the metabolism pathways are needed (Youngster et al., 2010). Additionally, none of the previous studies were able to track microbial community composition and activity from initial exposure to MTBE up to the point where MTBE biodegradation was observed.

OBJECTIVE

The broad objective of this research was to characterize how the microbial community responded to MTBE contamination in the native marine sediment. Furthermore, this research sought to identify specific microorganisms responsible for biodegradation of MTBE and specific pathways with or without engineering intervention. More specifically, the first objective was to quantify the native MTBE attenuation rates and identify processes that can increase the rate and extent of MTBE and TBA degradation. The second objective was to use metagenomic sequencing and high throughput 16S DNA gene sequencing to determine both the shifts in microbial populations and microbial activity related to the MTBE spill.

APPROACH

Marine sediment samples were collected from the Port of Houston at four different locations. Two locations were approximately the same geographical location as Morgan's Point where the spill occurred, and two additional locations were selected further downgradient of the shipping channel where MTBE was likely to travel due to boat traffic. In addition, six different locations from the Gulf of Mexico were provided by Battelle for background comparison. These six locations from Battelle provided data on the typical composition of marine microbial communities and allow for comparison to microbial communities that had been exposed to MTBE for several months. To characterize the microbial community, metagenomic analyses using Illumina MiSeq analysis was utilized. Metagenomic analyses via MiSeq was performed on extracted and

amplified DNA at the Clemson University Genomics Institute (CUGI) using the Illumina MiSeq series sequencer. Briefly, extracted DNA from collected samples were PCR amplified using primer pairs that were Illumina MiSeq tagged. The MiSeq sequencing was carried out according to the CUGI instructions.

Standard anoxic batch incubations were used to determine the rate and extent of MTBE degradation, and the projected pathway of biodegradation by analyzing known intermediates such as TBA. Standard anoxic, glass serum bottles sealed with a butyl rubber stopper and sampled with anoxic gas flushed syringe and needle were used for the batch incubations. MTBE, TBA, methane, and other volatile compounds were analyzed using a gas chromatograph with a flame ionization detector (GC-FID). Different ion concentrations, sulfate, chlorine, nitrate, and phosphate, were analyzed using an ion chromatograph to characterize the sampled media.

MATERIALS AND METHODS

Sample Collection

The four locations chosen for sampling can be seen in Figure 1. This map represents the approximate location of the four sites chosen.

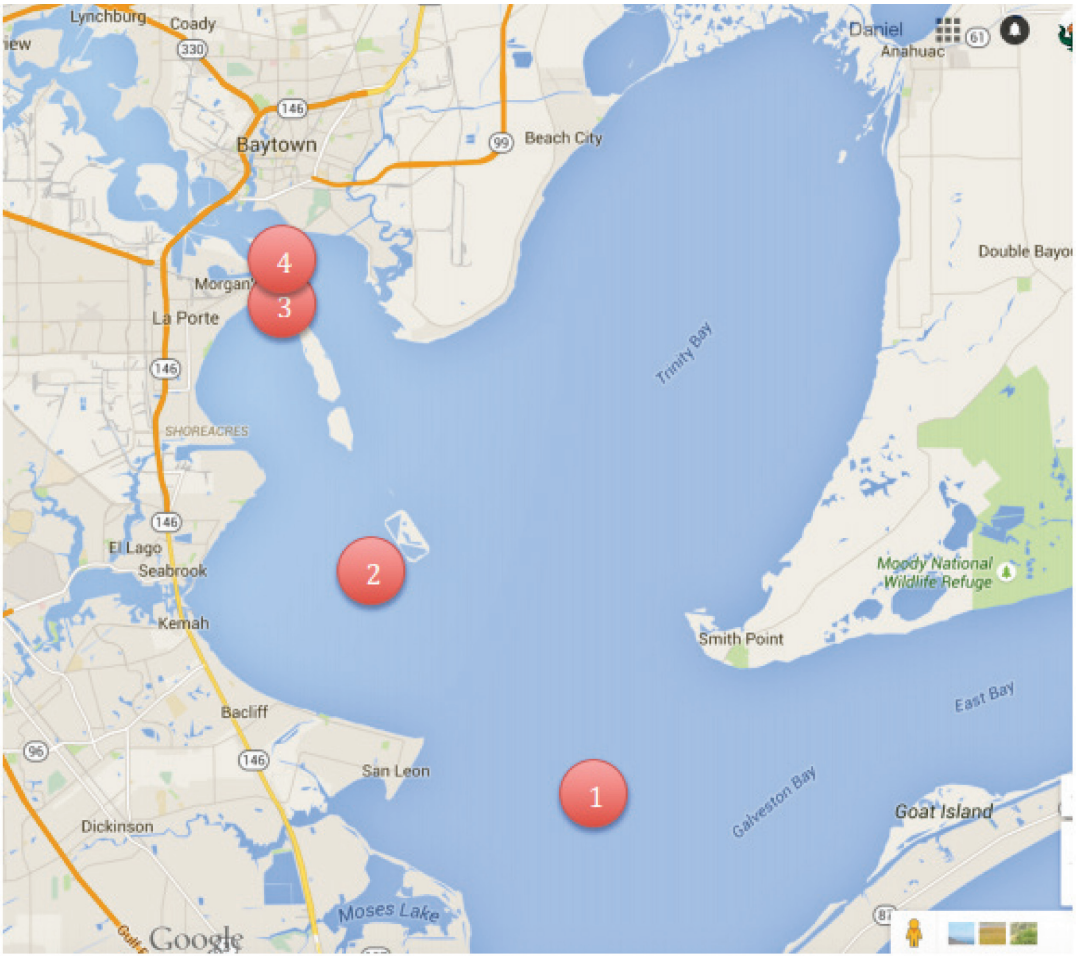

Figure 1. Map of sample locations show the approximate locations sampled for MTBE community analysis. Location 4 is appromately where the spill occurred and each location is located in the shipping channel heading towards the Gulf of Mexico.

The exact locations can be found in Table A-1 in Appendix A. For reference, the boat collision happened at approximately location 4. The sampling plan outline I used to collect samples can be seen in detail in Appendix B. Two quart sized mason jars of

sediment and four 50 mL Falcon centrifuge tubes were collected at each site. Briefly, an Eckman dredge was used to trap sediment and bring it to the boat. Once sediment was brought to the boat deck, the four Falcon tubes were filled quickly to the top. The lids were tightly fastened and the tubes were placed into a cooler of dry ice. Next, the remaining sediment was scooped into the mason jars. The sediemnt was settled every inch or two by tapping the jar. When reaching the top of the jar, the sediment was overpacked to form a meniscus at the top. After a few final taps, the lid was screwed down very tightly and placed into another cooler. Both coolers were then shipped overnight. The coolers were stored in the freezer until needed for analysis.

Batch Study

In order to observe if any biological attenuation with the native microbial community was possible, a batch study was set up using each of the four Houston sites and the six background sites from Battelle using previously reported strict anaerobic techniques (Wei and Finneran, 2011; Wei and Finneran, 2009). I amended each site with a different terminal electron acceptor and either MTBE or TBA as the amended electron donor. Before the batch study was set up in an anoxic glovebag, the glovebag was vacuumed and pumped with pure nitrogen three times and allowed to equilibrate for 30 minutes. Then, the sediment from each site were homogenized to ensure a full representation of the sample. Approximately 20 grams of soil were placed into 60 mL serum bottles and ATCC medium: 1958 Desulfuromonas medium, which represents modified marine water, was added to a total aqueous volume of 30 mL in order to saturate the sediment. The bottles were sealed with thick butyl rubber stoppers and

crimped. Once the sealed bottles were removed from the glovebag, the headspace was flushed with pure nitrogen gas, which had passed through a heated, reduced copper filled glass tube to remove any traces of oxygen. Before any bottles were amended with anything, one bottle from each site was initial scanned to see if any native MTBE or TBA could be detected. No sites had any MTBE and TBA above detection limit, meaning we had to manually spike each bottle with MTBE or TBA.

Each of the ten sites had a total of fourteen experiments conducted, seven which were MTBE amended (1 mM) and seven which were TBA amended (5 mM). Electron acceptors were added to stimulate different environmentally significant TEAP zones, which included 10 mM Fe(III), 10 mM nitrate, and 10 mM sulfate. Additionally, bottles from each site were unamended without any electron donor, amended with 10 mM fumarate, and amended with 10 mM Fe(III) + 500 μM antraquinone-2,6-disulfonate (AQDS). AQDS has been shown to accelerate bioremediation by serving as an electron shuttle between Fe(III) reducing bacteria and Fe(III) oxides (Finneran and Lovley, 2001). All reagents were added from anoxic, sterile stock solutions using anaerobic syringes. Control experiments were set up for each site by autoclaving at 121 °C for one hour/day, for three straight days. This was to observe any chemical reactions or reductions that may have occured through non-biological processes. A full experimental matrix can be seen in Appendix C.

MTBE and TBA degradation were determined using headspace analysis by gas chromatography (Shimadzu GC-2014) using a flame ionization detector and an Agilent Technologies HP-1 column that measured 30 m in length, 0.320 mm diameter, and 0.25

μm film (Part Number 19091Z-413E). The carrier gas was ultra-pure nitrogen (2.10 mL/min) and the oven temperature was 40 °C for five minutes before ramping up to 100 °C at a rate of 40 °C/min. The injection port temperature was 250 °C and the detector temperature was 260 °C. The injection procedure used for the batch study included taking a 0.5 mL VICI Precision Sampling, Inc. Pressure-Lok Precision Analytical Syringe and flushing it with pure nitrogen gas that had been passed through a heated copper wire. The syringe was filled to 0.2 mL of nitrogen and then injected into the sample bottle. The headspace of the sample was flushed three times before removing 0.2 mL of headspace from the bottle and letting it equilibrate for five seconds. The syringe was then locked, removed from the sample, and the Glide needle was replaced with the injection needle. The syringe was then injected into the port of the GC. In between samples, the syringe and injection needle were placed on a vacuum system until the next sample was ready.

Approximately 50 days into the initial batch study, methane generation occurred to the point where a methane peak from the FID engulfed the MTBE and TBA peaks. This made it impossible to determine the MTBE and TBA concentrations and identify if any degradation had occurred. While trying to discover a solution, four of batch bottles which showed the most degradation action were selected to conduct a Most Probable Number (MPN) experiment. The MPN approach was designed with the intention of isolating the methane producing organisms using a series of 10% dilutions to fresh modified marine media. This approach has been used successfully before (Alexander, 1982; Yeh and Novak, 1994). Dilutions for each of the four bottles were carried out to 10E-06 in 60 mL serum bottles. The four bottles selected were the Houston MTBE and

Fe(III), Battelle MTBE and sulfate, Houston TBA and sulfate, and the Battelle and fumarate. Approximately 27 mL of ATCC: 1958 media were added anaerobically to each bottle before approximately 3 mL transfers were added. Each bottle was amended with 5 mM Fe(III), 5 mM nitrate, 5 mM sulfate, and 5 mM MnO_2 as electron acceptors.

The solution to continue the initial batch studies was to add 20 mM 2-bromoethanesulfonate (BES) which has shown to inhibit methanogenesis while having no effect on MTBE and TBA degradation (2002NJ6B, 2002). This was only utilized for the Houston MTBE and Houston TBA batches. The first step was to attempt to remove all the methane from each bottle by flushing the headspace with pure nitrogen for up to one hour. Next, the BES and electron acceptors (to initial concentrations) were added anaerobically. Finally, the bottles were re-spiked with either 1 mM MTBE or 5 mM TBA.

DNA Extraction Method

Prior to DNA extraction, each sample was separately homogenized in an anoxic, nitrogen filled glove bag. This was to ensure that the DNA sample used was representative of the entire community. The DNA was extracted using an UltraClean® Soil DNA Isolation Kit by MO BIO Laboratories, Inc. (Cat. No. 12800-100). The protocol was given by the manufacturer. Approximately one gram of soil was taken from each sample and added to separate 2 mL Bead Solution Tubes. The bead solution is a buffer that disperses the soil particles and begins to dissolve humic acids. After gently inverting the tubes to mix the sample and the bead solution, 60 µL of Solution S1 was added to the tube and vortexed briefly. The tubes were vortexed using a Baxter Scientific

Products SP® Vortex Mixer (Cat. No. S8223-1). Solution S1 is a detergent that contains sodium dodecyl sulfate which aids in cell lysis. The detergent breaks down the fatty acids and lipids interrelated within the cell membrane of the present organisms. Solution S1 was heated to 60°C until all precipitates dissolved before use using a Fisher Scientific Isotemp 202S water bath (Cat. No. 15-462-3SQ). To each tube 200 µL of Inhibitor Removal Solution (IRS) was then added. IRS is a reagent that precipitates humic acids and other PCR inhibitors so that they can be removed. This step was required because the extracted DNA was to be used for PCR. All bead tubes were then secured horizontally on a flat-bed Fisher Vortex Genie 2™ made by Fisher Scientific (Cat. No. 12-812) and vortexed at maximum speed for 15 minutes for mechanical cell lysis. The 2 mL Bead Solution Tubes were then centrifuged at 10,000 x g for 30 seconds using a VWR® Micro 2416 Microcentrifuge (Cat. No. 37001-300). All particulates including cell debris, soil, beads, and humic acids formed a pellet at the bottom of the tube and the DNA formed a liquid supernatant. The supernatant was transferred to a clean 2 mL collection tube provided in the extraction kit. Approximately 600 µL of supernatant was produced in each sample. Next, 250 µL of Solution S2 was added. Solution S2 contains a protein precipitation reagent that removes contaminating proteins that may reduce DNA purity and inhibit future PCR. The tubes were then vortexed for five seconds and incubated at 4°C for five minutes. All tubes were then centrifuged for one minute at 10,000 x g. The entire volume of supernatant, making sure to avoid the pellet at the bottom of each tube, was transferred to a new clean 2 mL collection tube. Approximately 900-1000 µL of supernatant was produced in each tube. Solution S3 (1.3 mL) was then added and

vortexed for five seconds. Solution S3 is a DNA binding salt solution and must be shaken thoroughly to mix solution before use. Solution S3 causes DNA to bind to silica in the presence of high salt concentrations. Next, 700 µL of mixture was loaded onto a provided Spin Filter and centrifuged for one minute at 10,000 x g. The flow through was discarded and the remaining supernatant was added to the Spin Filter and centrifuged at 10,000 x g for one minute. This step was repeated until all supernatant had passed through the Spin Filter. Depending on the yield, it took either three or four loads for each sample. In this step, DNA bound to the silica membrane in the spin filter and almost all contaminants passed through the filter membrane, leaving only the desired DNA left behind. Then, 300 µL of Solution S4 were added and the tubes were centrifuged for 30 seconds at 10,000 x g. The flow through was discarded and the tubes were centrifuged again at 10,000 x g for one minute. Solution S4 is an ethanol based wash solution that further cleans the DNA that is bound to the silica membrane in the spin filter. The wash removes residues of salt, humic acid, and other contaminants while leaving DNA intact. The spin filter was taken out of the collection tube and placed into a new collection tube. Fifty µL of Solution S5 was placed in the center of the filter membrane and centrifuged at 10,000 x g for 30 seconds. Solution S5 is a sterile elution buffer (10 mM Tris pH 8.0) that releases DNA as it passes through the silica membrane and into the collection tube. The DNA was released because it can only bind to the silica spin filter membrane in the presence of salt (UltraClean® Soil DNA Isolation Kit Instruction Manuel). The spin filter was discarded and the DNA in the tube was then ready to determine concentrations and to be used for PCR applications.

Concentration was determined using Thermo Scientific™ Nanodrop 200/2000c Spectrophotometer (Cat. No. ND2000). The Nucleic Acid application within the program was used to determine the nucleic acid concentration and purity. A default wavelength of 340 nm was used for a bichromatic normalization. A blank was established by loading 2 μL of nuclease free water, obtained from Qiagen (Cat. No. 129117), onto the lower measurement pedestal, lowering the sampling arm and selecting blank. Once the measurement was complete, the sampling arm was raised and the sample was wiped from both the upper and lower pedestals using a dry Kimwipe. From there, each DNA extraction sample was loaded onto the lower pedestal and measured. Each sample was measured using the DNA-50 type, which is used for double stranded DNA. The significance of the results came from the concentration and the 260/280 ratio. The concentration is based on the absorbance at 260 nm and is given in units of ng/μL. The 260/280 ratio is the ratio of absorbance at 260 nm and 280 nm. This is used to assess the purity of DNA. A ratio of approximately 1.8 is considered as "pure" DNA (NanoDrop 2000/2000c Spectrophotometer V1.0 User Manual, 2009). In the event that extracted DNA did not work correctly or was used up during unsuccessful PCR runs, the original samples would be re-extracted following the same protocol. Data on extracted concentrations and 260/280 ratios are found in Table D-1 in Appendix D.

PCR Amplification, Purification, and Quantification

PCR was conducted using two QIAGEN kits, Taq DNA Polymerse (Cat. No. 201205) and Taq PCR Core Kit (Cat. No. 201225). These kits contain buffers and reagents that are critical for successful amplification. Three different Illumina MiSeq

tagged primer pairs, two bacterial and one archaeal, were used. Each primer and tag sequences can be found in Appendix E. Using three different primer pairs allowed for complete screening of the microorganism community present. The two bacterial primer pairs, 341F/785R and 338F/907R, have been reported to achieve high overall bacterial coverage with reliable accuracy (Klindworth et al., 2012). The use of two bacterial primers reduces the possibility of excluding certain bacteria present that may not be ideally amplified using a particular primer pair. Additionally, it also provides more conclusive evidence of potentially significant microbial groups if the pattern is present in the data from both primer pairs. The archaeal primer pair was developed by Suzuki et al (2000). This particular primer pair uses a mix of two forward primers and has been used successfully for enhancing anaerobic degraders (Silva and Alvarez, 2004). The PCR reaction setup was provided by Clemson University Genomics Institute (CUGI) and can be found in Appendix F. Thermocycler settings were 94 °C for 10 minutes for initial denaturing, then 39 cycles of 94 °C denaturation for 1 minute, 55 °C (50 °C for archaeal primer) annealing for 1 minute, 72 °C extension for 1 minute, followed by a final extension time of 10 minutes at 72 °C.

After PCR was completed, each sample plus a negative control were checked by gel electrophoresis. A 1% agarose gel in 1x TAE buffer was prepared while samples were running in the thermocycler. Once the gel was solidified, the gel was placed in the gel box containing 1x TAE buffer so that the buffer was covering the gel five to ten millimeters above the gel. Five µL of each sample plus the negative control were mixed with 1 µL of loading dye. Five µL of the mixture was carefully loaded into an open well.

Power was supplied at a voltage of 15 V for ten minutes, then increased to 70 V for 50 minutes. After completion, the gel plate was placed on a UV transilluminator to confirm the presence of PCR product by checking for illuminated bands in the correct target range. Correct target ranges are found by subtracting the forward primer value from the reverse primer values. If amplification was unsuccessful, PCR would have to be re-ran. Examples of successful PCR amplification for each primer pair can be seen in Appendix G.

After successful amplification of the target sequence, the samples were purified using Qiagen QIAquick® PCR Purification Kit (Cat. No. 28106). The manufacture protocol for purification using a microcentrifuge was followed. Five volumes of Buffer PB were added to one volume of the PCR sample and was mixed by pipetting. Buffer PB enables efficient binding of double-stranded PCR products to the spin column membrane. The mixture was then loaded onto a QIAquick spin column and centrifuged for one minute. After discarding the flow through and placing the column back in the same tube, 0.75 mL of Buffer PE was added and the samples were centrifuged for one minute. Buffer PE is a wash buffer that removes unwanted primers and impurities. The QIAquick column was then placed in a clean 2 mL microcentrifuge tube and 30 µL of Buffer EB was added to the center of the membrane and centrifuged for one minute. Buffer EB is an elution buffer of 10 mM Tris-Cl that allows DNA to pass through the membrane and into the collection tube (QIAquick PCR Purification Kit Protocol, 2008).

DNA concentrations in each sample were determined using the ThermoFisher Scientific dsDNA HS (high sensitivity) Assay Kit (Cat. No. Q32851) and a Qubit® 2.0

Fluorometer (Cat. No. Q32866). This assay is highly selective for double-stranded DNA (dsDNA) and is highly accurate. The assay was performed based on the manufacturer's instructions. Briefly, a working solution was created for two standards and each sample by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. Each of the two standards were made by combining 190 µL of the working solution with 10 µL of each respective Qubit® Standard. To each tube 198 µL of working solution were added. Two µL of purified PCR product from each sample were added to each of the working solution tubes. After a quick vortex, the samples were incubated at room temperature for two minutes. All standards and samples were then read on the fluorometer (MAN0002326, 2015). Concentrations were normalized to a final concentration of 30 ng/µL using TE buffer as a diluent and given to CUGI for analysis.

Sequence Analysis

The normalized PCR amplicons will be sequenced via next-generation, massively parallel, high throughput Illumina MiSeq Sequencing. High throughput analysis is basically a random sequencing approach in which small base pair contiguous units are generated and compared against publically available databases to predict the total number of phylotypes present and the relative abundance of each phylotype. Sequences will trimmed and aligned using an Illumina BaseSpace account. BaseSpace has the ability to generate summary reports for each sample amplified for each primer and categorize each reading into Kingdom, Phylum, Class Order, Family, Genus, and Species. Statistical analysis of taxonomic and functional profiles (STAMP) software will be used to assess biological importance of microbial signatures of 16S DNA. Illumina sequencing is a

proven, well established technology producing over 90% of all sequencing data and referenced in 4,800 peer-reviewed publications (MiSeq Sequencing System, 2016). Illumina's sequencing is the most successful and widely-adopted next-generation sequencing technology worldwide (MiSeq Sequencing System, 2016).

RESULTS AND DISCUSSION

Batch Study

Results from the Houston MTBE, TBA, and MPN data can be found in Appendix H. Looking at the Figure I-1, the Houston MTBE control for the four sites, it can be seen that each concentration after 55 days were either equal or greater than 100%. This indicates that no chemical reactions resulting in non-biological degradation occurred. The same can be said for the Houston TBA control set as seen in Figure I-8. Although the concentrations fluctuated over time, after 55 days all concentrations were nearly 100% indicating no degradation activity occurred. For each of the eight bottles that were not amended with an electron acceptor, only site four Houston MTBE spiked bottled showed any sign of degradation after 55 days. The final concentration was approximately 80% of the initial concentration. The results of unamended bottles can been seen in Figure I-2 and Figure I-9.

For the Houston MTBE and fumarate amended bottles, sites three and four appeared to have some degradation activity after 27 and 41 days, respectfully, but both bottles increased at the next time point of 55 days. It is unclear if this is a result of technique or instrumental error. Sites one and two did not appear to have much activity,

remaining at concentrations above 90%. Those results are seen in Figure I-3. Figure I-10, the Houston TBA and fumarate bottles from sites one, three, and four showed degradation activity while site two does not show much activity remaining consistently around 90% of the initial concentration. Site one was at nearly 100% initial concentration after 21 days but the concentration decreased linearly the next 14 days to 78% of the initial. Site three and four both showed degradation at nearly the same rate since day 0, with larger degradation rates until day 21. After day 21, degradation at sites three and four decreased and remained at concentrations of 71% and 64%, respectfully, producing a degradation rate of -0.6545 mM/day for site four. No studies could be found using fumarate as the only electron donor source.

All four sites for the Houston MTBE and Fe(III) and Houston TBA and Fe(III) amended bottles remained at a concentration near 80%, although each bottle had a steady linearly decreasing concentration over the 55 days as seen in Figure I-4 and Figure I-11. None of the Houston MTBE Fe(III) and AQDS amended bottles showed degradation activity until 27 days in. Then, sites one, three, and four all had concentrations decrease at the same linear rate through day 41. None of the concentrations from those three sites were less than 80% of the initial concentration and each slightly increased on day 55. No degradation occurred in the site two bottle. These results are displayed in Figure I-5. The Houston TBA Fe(III) and AQDS amended bottles all showed linear degradation at similar rates from day 0 to day 21 to a concentration approximately 80% of the initial concentration. After day 21, no further degradation occurred in any bottle as seen in Figure I-12. It is interesting to note that although AQDS is known to facilitate

biodegradation, MTBE concentrations in Fe(III) amended bottles were nearly equal between AQDS and non-AQDS amended bottles. However, AQDS amended bottles had lower concentrations compared to the Fe(III) amended bottles without AQDS for the TBA study.

In Figure I-6, the Houston MTBE and nitrate bottles showed mixed results. Sites two and four remained at approximately 100% of the initial concentration while sites one and three both degraded at nearly the same rate to a final concentration of 79% and 76%, respectively, of initial concentrations. The Houston TBA and nitrate amended bottles all showed some degradation activity as seen in Figure I-13. Sites one, two, and four all degraded to approximately 75% of their initial TBA concentration. Site three degraded much quicker initially than the other three sites and dropped to a concentration of 72% after just seven days. After the seven days, the concentration decreased at a much lower rate to a final concentration of 66% after the 55 days.

The Houston MTBE and sulfate amended bottles for sites one, two, and three showed slight degradation to final concentrations of 85%, 84%, and 81%, respectively. Site one showed the greatest initial degradation, site two had the slowest initial degradation, and site three fell in between the two sites. Site four did not show any degradation activity after 55 days which is interesting given that site four is the location that the spill took place. The results are seen in Figure I-7. The Houston TBA and sulfate bottles showed the largest amount of degradation activity, seen in Figure I-14. Sites two, three, and four each showed the most degradation activity after 13 days. Site two

decreased to 73%, site three to 48%, and site four to 59% after the 13 days then remained at those concentrations through the 55 days.

Figure I-15, the Houston MTBE MPN dilution series showed no degradation activity after 42 days before five of the six dilutions showed a sharp decrease in final concentration by day 53. While the dilution of 10E-05 remained at a concentration of 88%, the dilutions of 10E-02, 10E-03, 10E-04, and 10E-6 ranged from 68-71%. Dilution 10E-01 fell to a final concentration of 63%. Results from the Battelle MTBE dilution series, Figure I-16, presented fluctuations in concentration for all the bottles except the 10E-05 dilution. The 10E-01, 10E-02, and 10E-05 dilutions all reached a concentration of 69-72% after the 53 days. The 10E-3 and 10E-6 dilutions reached final concentrations of 81% and 79%, respectfully. The 10E-4 dilution showed no activity remaining at a concentration of 95% of the initial concentration after the 55 days.

The Houston TBA and sulfate site 3 amended dilution series, Figure I-17, also had high levels of fluctuation over the span of 55 days. All the dilution bottles except for 10E-05 hovered at concentrations above 100% for the majority of the experiment time, but the bottles eventually reached a concentration of 84% or less. The 10E-01, 10E-04, and 10E-05 dilutions ranged from 82-84%, while the 10E-02 and 10E-06 dilutions finished at a concentration of 76% and 74%, respectfully. Dilution 10E-03 had the lowest final concentration at 69% of the initial concentration. Figure I-18, the Battelle TBA and fumarate amended dilution from site 6 had the least amount of collective activity of all the MPN batch studies. The 10E-04 and 10E-06 bottles failed to reach 90% of the initial concentrations after 55 days. Dilutions 10E-01, 10E-3, and 10E-5 showed slightly more

degradation activity and had a final concentration that ranged from 80-82%. The 10E-02 dilution showed the most degradation activity of this set by finishing at a concentration of 70%. As with the other three MPN studies, most degradation occurred between days 42 and 55.

Overall, results from all batch experiments are extremely encouraging. While biodegradation has been observed for all relevant terminal electron acceptors (excluding TBA biodegradation under methanogenic conditions), majority of batch experiments involve an initial incubation period of hundreds of days (Schmidt et al., 2004). Additionally, majority of all known studies have been conducted using fresh surface-water sediment and fresh water aquifer sediment. The fact that some degradation activity has occurred after 53 or 55 days indicates that continuous monitoring of these batch studies is needed.

While Somsamak et al. (2001) did not observe any MTBE degradation under Fe(III) conditions in estuarine sediment, two other studies did show mineralization of MTBE in surface water sediment and aquifer sediment (Finneran and Lovley, 2001; Bradley et al., 2001). However, Bradley et al. (2001) took 166 days to reach 14% mineralization and Finneran and Lovley (2001) observed 19-30% mineralization in 130 days, after 300 days pre-incubation. Finneran and Lovley (2001) also observed greater than 25% reduction in TBA. For the Houston and Fe(III) amended site three, a zero order degradation rate of -0.4364 mM/day. Site three also produced a degradation rate of -0.4545 for TBA and Fe(III) amended bottles.

Bradley et. al (2002) reported 49% mineralization of TBA in surface water sediment after 198 days under denitrifying conditions. Bradley et al. (2001) also reported 23-75% mineralization of MTBE under denitrying conditions after 166 days. However, no MTBE degradation occurred in an estuarine sediment after 1160 days (Somsamak et al., 2001). In the batch study set up in this thesis, rates of -0.3818 mM/day and -0.4364 mM/day for Houston sites one and three, respectively, for MTBE and nitrate amended bottles. Borden et al., (1997) also didn't observe any MTBE degradation after 263 days.

Like other terminal electron acceptors mentioned, sulfate reductions contain mixed results that are site specific. Using a soil sediment no MTBE degradation was observed after 280 days while some TBA degradation (unspecified amount) was observed (Yeh and Novak, 1994). Bradley et. al (2001) reported 9-20% mineralization of MTBE after 166 days. Bradley et al. (2002) also reported 5% mineralization after 198 days.

Sequencing

Sequencing data will be made available once samples have been returned from CUGI.

CONCLUSIONS

As indicated above, results from this batch study are encouraging and should be continually monitored. Fifty five days is not a long enough period to deem a specific culture as degrading, but given the rate of degradation in some if not most of the bottles indicates that degradation of native cultures is certainly possible. As cultures capable of

degradation continue to grow, enrichment transfers should be used to isolate specific strains of bacteria responsible for the degradation. This will allow the culture to thrive with the energy sources they need. From there DNA extractions and amplifications can be conducted to indicate which microbes are responsible. It is interesting to note that of all four Houston sites, site three appears to be the most active. The first possibility is that this site was more enriched with degraders when sampled. Only having one bottle for each electron acceptor for each site limits the ability to fully understand what is taking place. Had this experiment been conducted with duplicate or triplicates, error bars could have been represented to justify with sites were none selective. Additionally, it is possible that site three had the highest exposure to MTBE. Given MTBE's hydrophilic nature, it is possible majority of the MTBE was away from site four and towards site three before it settled. Therefore, site three would have had the most exposure causing the microorganisms to have more time to adapt. It would be interesting to set up experiments in triplicates and test this theory. Also future work could include going back to the same locations and pulling more samples. Having had a year to adapt, it is interesting to see how native microorganisms changed.

When the results of the sequencing data is made available it will be interesting to look at three different comparisons. First, comparing the results from the four Houston sites with the six non-contaminated Battelle sites. It will be interesting to see how the entire microbial community in the Port of Houston responded after MTBE exposure. Secondly, it will be interesting to compare Houston sites three and four with Houston sites one and two. Sites three and four happened close to where the actual spill occurred,

while sites one and two happened further down the stream of the channel. If sites three and four had more exposure to MTBE than it is possible their microbial community responded differently than sites one and two whom may have had less exposure. Third, it would be interesting to do DNA extractions on batch study bottles that have shown degradation and compare them to the original Houston samples. This will allow a look at which microorganisms are thriving and which species may be responsible for the degradation.

APPENDICES

Appendix A

Sample Locations

Table A-1. This table indicates the exact latitude and longitude of the four sampling sites in the Port of Houston.

Location	Latitude	Longitude
1	29° 27’ 31.0” N	94° 50’ 59” W
2	29° 33’ 29.9” N	94° 55’ 03.8” W
3	29° 39’ 13.1” N	94° 58’ 22.2” W
4	29° 41’ 03.4” N	94° 58’ 49.7” W

Appendix B

Sampling Procedure

Sampling procedure (PROVIDED BY: Kevin Finneran – Clemson University)
Marine sediment
Houston, TX (Port of Houston)

Per each sampling location (four (4) total) you will collect:

- 2 large jars of sediment
- 4 Falcon tubes for frozen molecular analyses

To collect large jars:

- They will have an Eckman or Ponar dredge; this is a long spring-loaded toothed bucket that drops to the bottom and a trigger snaps it shut with the sample inside; water runs out so you get solids
- Pull it on to the boat
- Begin scooping into the jar and tap the jar to settle the sediment every inch or two (i.e. pack it down)
- When you get near the top of the jar start letting the material overflow the sides so it forms a meniscus at the top and it is packed in
- Give a few final taps and then using the lid press down on to the sediment so it flows out under the sides and runs down the container
- Use paper towels to wipe the threads super clean (water too if you need it; bring a squirt bottle)
- While holding down the lid screw down the top very tightly
- Place a nitrile glow over the top
- Put in the cooler and make sure you label the lid before sealing it up
- TWO jars per location is all we need
- Ship overnight back to Rich lab

To collect frozen molecular samples:

- Get dry ice at a grocery store and crush it up in the smallest cooler
- Label all sample containers (4 per location)
- Before you get the large jar sample fill each of these quickly to the top but don't worry about anoxia
- Screw the lid tightly and jam down into the dry ice
- Move quickly
- FOUR per location
- Ship overnight back to Rich lab; make sure FedEx knows there is dry ice in the cooler

For sampling:

- Bring 1 whole case of water (24 bottles); drink one every 30 minutes you are out there
- Wear "fishing clothing" (i.e. light but covering your arms if possible)
- Sunblock – like you mean it!
- Use caution on the boat and listen to the Captain and crew
- BRING YOUR TWIC CARD!!!

KTF

Appendix C

Experimental Matrix

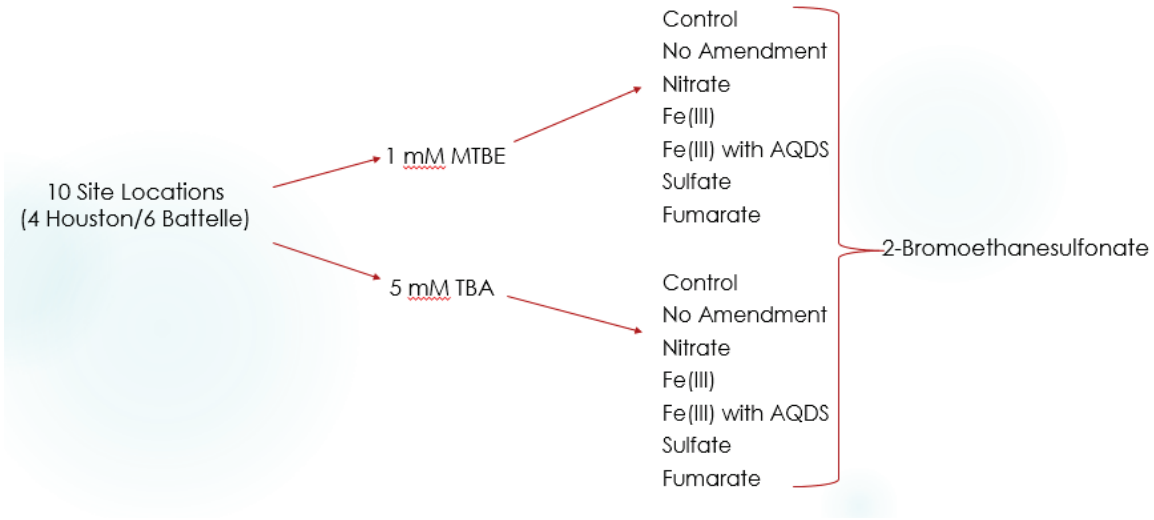

Figure C-1. A visual representation of the initial experimental matrix. Only the four Houston sites were studied with 2-bromoethanesulfonate.

Houston			
MTBE			
Site 1	Site 2	Site 3	Site 4
Kill Control	Kill Control	Kill Control	Kill Control
Unamended	Unamended	Unamended	Unamended
10 mM Fumarate	10 mM Fumarate	10 mM Fumarate	10 mM Fumarate
10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)
10 mM Fe(III) + 500 µM AQDS	10 mM Fe(III) + 500 µM AQDS	10 mM Fe(III) + 500 µM AQDS	10 mM Fe(III) + 500 µM AQDS
10 mM Nitrate	10 mM Nitrate	10 mM Nitrate	10 mM Nitrate
10 mM Sulfate	10 mM Sulfate	10 mM Sulfate	10 mM Sulfate

Houston			
TBA			
Site 1	Site 2	Site 3	Site 4
Kill Control	Kill Control	Kill Control	Kill Control
Unamended	Unamended	Unamended	Unamended
10 mM Fumarate	10 mM Fumarate	10 mM Fumarate	10 mM Fumarate
10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)
10 mM Fe(III) + 500 μM AQDS	10 mM Fe(III) + 500 μM AQDS	10 mM Fe(III) + 500 μM AQDS	10 mM Fe(III) + 500 μM AQDS
10 mM Nitrate	10 mM Nitrate	10 mM Nitrate	10 mM Nitrate
10 mM Sulfate	10 mM Sulfate	10 mM Sulfate	10 mM Sulfate

Battelle Background					
MTBE					
Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Kill Control	Kill Control	Kill Control	Kill Control	Kill Control	Kill Control
Unamended	Unamended	Unamended	Unamended	Unamended	Unamended
10 mM Fumarate	10 mM Fumarate	10 mM Fumarate	10 mM Fumarate	10 mM Fumarate	10 mM Fumarate
10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)
10 mM Fe(III) + 500 μM AQDS	10 mM Fe(III) + 500 μM AQDS	10 mM Fe(III) + 500 μM AQDS	10 mM Fe(III) + 500 μM AQDS	10 mM Fe(III) + 500 μM AQDS	10 mM Fe(III) + 500 μM AQDS
10 mM Nitrate	10 mM Nitrate	10 mM Nitrate	10 mM Nitrate	10 mM Nitrate	10 mM Nitrate
10 mM Sulfate	10 mM Sulfate	10 mM Sulfate	10 mM Sulfate	10 mM Sulfate	10 mM Sulfate

Battelle Background

TBA

Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Kill Control	Kill Control	Kill Control	Kill Control	Kill Control	Kill Control
Unamended	Unamended	Unamended	Unamended	Unamended	Unamended
10 mM Fumarate	10 mM Fumarate	10 mM Fumarate	10 mM Fumarate	10 mM Fumarate	10 mM Fumarate
10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)	10 mM Fe(III)
10 mM Fe(III) + 500 µM AQDS	10 mM Fe(III) + 500 µM AQDS	10 mM Fe(III) + 500 µM AQDS	10 mM Fe(III) + 500 µM AQDS	10 mM Fe(III) + 500 µM AQDS	10 mM Fe(III) + 500 µM AQDS
10 mM Nitrate	10 mM Nitrate	10 mM Nitrate	10 mM Nitrate	10 mM Nitrate	10 mM Nitrate
10 mM Sulfate	10 mM Sulfate	10 mM Sulfate	10 mM Sulfate	10 mM Sulfate	10 mM Sulfate

Appendix D

Extracted DNA Data

Table D-1. This table displays all data related to DNA extraction. It includes the sample ID, time, DNA concentration (Nucleic Acid Conc.), and purity (260/280).

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	HB1	gel box	10/26/2015 15:55	38.4	ng/µl	0.77	0.43	1.8	1.14	DNA	50
2	HB2	gel box	10/26/2015 15:58	25	ng/µl	0.5	0.29	1.74	0.77	DNA	50
3	HB3	gel box	10/26/2015 15:59	58.7	ng/µl	1.18	0.64	1.83	1.18	DNA	50
5	HB4	gel box	10/26/2015 16:01	29.4	ng/µl	0.59	0.35	1.68	0.71	DNA	50
7	HF1_1	gel box	10/27/2015 18:51	14.8	ng/µl	0.3	0.17	1.72	0.6	DNA	50
8	HF1_2	gel box	10/27/2015 18:51	35.6	ng/µl	0.71	0.38	1.86	0.19	DNA	50
10	HF1_3	gel box	10/27/2015 18:52	11.8	ng/µl	0.24	0.12	2.05	0.19	DNA	50
11	HF1_4	gel box	10/27/2015 18:53	11.1	ng/µl	0.22	0.11	1.96	0.31	DNA	50
12	HF2_1	gel box	10/27/2015 18:53	17.6	ng/µl	0.35	0.19	1.83	0.67	DNA	50
13	HF2_2	gel box	10/27/2015 18:54	13.5	ng/µl	0.27	0.15	1.78	0.6	DNA	50
14	HF2_3	gel box	10/27/2015 18:54	15.9	ng/µl	0.32	0.19	1.68	0.58	DNA	50
15	HF2_4	gel box	10/27/2015 18:54	22.9	ng/µl	0.46	0.25	1.85	0.27	DNA	50
16	HF3_1	gel box	10/27/2015 18:55	19.6	ng/µl	0.39	0.21	1.85	0.67	DNA	50
17	HF3_2	gel box	10/27/2015 18:55	14.2	ng/µl	0.28	0.14	1.97	0.68	DNA	50
18	HF3_3	gel box	10/27/2015 18:56	18.2	ng/µl	0.36	0.21	1.74	0.55	DNA	50
19	HF3_4	gel box	10/27/2015 18:56	19	ng/µl	0.38	0.21	1.82	0.64	DNA	50
22	HF 4-2	gel box	10/28/2015 15:14	17.2	ng/µl	0.35	0.22	1.58	0.42	DNA	50
23	HF 4-3	gel box	10/28/2015 15:15	33	ng/µl	0.66	0.42	1.59	0.39	DNA	50
24	HF 4-4	gel box	10/28/2015 15:15	32.6	ng/µl	0.65	0.43	1.52	0.43	DNA	50
25	HF 4-1	gel box	10/28/2015 15:16	34.4	ng/µl	0.69	0.44	1.56	0.46	DNA	50
1	B1	gel box	12/5/2015 15:40	23.4	ng/µl	0.47	0.28	1.65	0.45	DNA	50
2	B2	gel box	12/5/2015 15:42	12.4	ng/µl	0.25	0.12	2.07	0.76	DNA	50
3	B3	gel box	12/5/2015 15:43	7.7	ng/µl	0.15	0.08	1.95	0.58	DNA	50
4	B4	gel box	12/5/2015 15:43	11.3	ng/µl	0.23	0.11	2	0.73	DNA	50
5	B5	gel box	12/5/2015 15:44	12.1	ng/µl	0.24	0.13	1.92	0.7	DNA	50
6	B6	gel box	12/5/2015 15:45	8.5	ng/µl	0.17	0.09	1.96	0.61	DNA	50
7	HF4_3	gel box	12/5/2015 15:46	23.1	ng/µl	0.46	0.29	1.6	0.48	DNA	50
8	HF4_3	gel box	12/5/2015 15:46	27.7	ng/µl	0.56	0.35	1.6	0.47	DNA	50
1	HB1	gel box	12/11/2015 22:55	3.1	ng/µl	0.06	0.02	2.87	0.04	DNA	50
2	HB2	gel box	12/11/2015 22:55	5.4	ng/µl	0.11	0.05	2.29	0.11	DNA	50
3	HB2	gel box	12/11/2015 22:56	1.3	ng/µl	0.03	0	10.66	0.02	DNA	50
4	HB4	gel box	12/11/2015 22:56	5.2	ng/µl	0.1	0.06	1.87	0.02	DNA	50
1	HB1	gel box	12/14/2015 17:18	22.4	ng/µl	0.45	0.25	1.78	0.93	DNA	50
2	HB3	gel box	12/14/2015 17:18	17	ng/µl	0.34	0.21	1.66	0.87	DNA	50
3	1_1	gel box	12/14/2015 17:19	7.7	ng/µl	0.15	0.09	1.73	0.36	DNA	50
4	1_3	gel box	12/14/2015 17:20	10.3	ng/µl	0.21	0.11	1.97	0.29	DNA	50
5	1_4	gel box	12/14/2015 17:20	8.6	ng/µl	0.17	0.11	1.57	0.56	DNA	50
6	2_1	gel box	12/14/2015 17:21	6.8	ng/µl	0.14	0.08	1.77	0.48	DNA	50
7	2_2	gel box	12/14/2015 17:21	12.7	ng/µl	0.25	0.14	1.82	0.76	DNA	50
8	2_3	gel box	12/14/2015 17:22	12.9	ng/µl	0.26	0.13	2.01	0.78	DNA	50
9	3_1	gel box	12/14/2015 17:22	15.5	ng/µl	0.31	0.18	1.72	0.71	DNA	50
10	3_2	gel box	12/14/2015 17:24	12.5	ng/µl	0.25	0.13	1.99	0.53	DNA	50
11	3_3	gel box	12/14/2015 17:24	8.1	ng/µl	0.16	0.1	1.68	0.53	DNA	50
12	3_4	gel box	12/14/2015 17:25	10.3	ng/µl	0.21	0.11	1.82	0.65	DNA	50

Appendix E

Primer Pairs

Primer	Sequence
341F	5'-***TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*** CCTACGGGNGGCWGCAG-3'
785R	5'-***GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*** GACTACHVGGGTATCTAATCC-3'
338F	5'-***TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*** ACTCCTACGGGAGGCAGC-3'
907R	5'-***GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*** CCGTCAATTCCTTTGAGTTT-3'
Arch1_1369F	5'-***TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*** CGGTGAATACGTCCCTGC-3'
Arch2_1369F	5'-***TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*** CGGTGAATATGCCCCTGC-3'
Arch1541R	5'-***GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*** AAGGAGGTGATCCTGCCGCA-3'

Appendix F

Illumina MiSeq PCR Reaction Setup

Components	**Amount per 1 Sample (µL)**
H2O (Nuclease free)	16
Q buffer	10
MgCl2	6
10x buffer	5
dNTP mix	4
Forward Primer	1.25
Reverse Primer	1.25
Borine Serum Albumin (BSA)	1
Extracted DNA	5
Taq Polmerase	0.5

Appendix G

Successful PCR Amplification

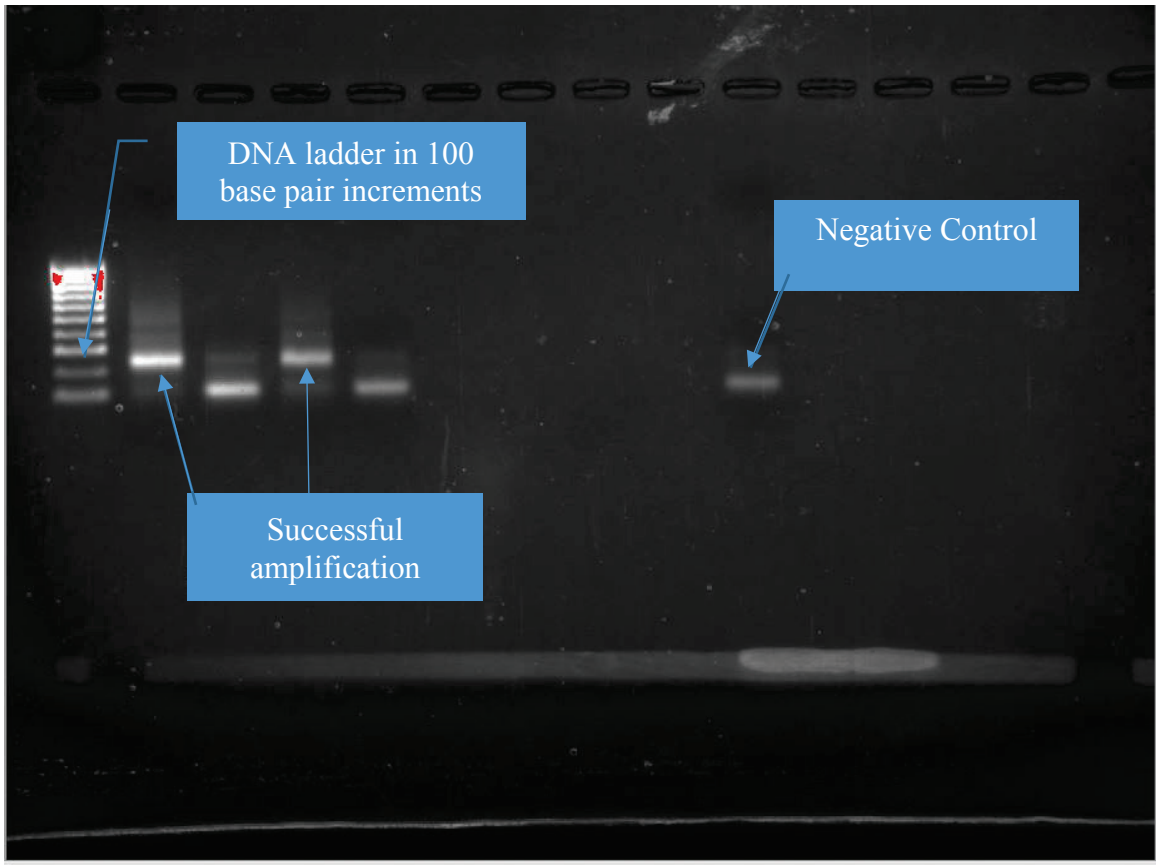

Figure G-1. This represents what a successful 1369F/1541R amplification looks like. Successful amplification would fall in the range of 200-300 based on the DNA ladder in lane 1. If bands are not reproduced in this range then PCR was unsuccessful. A negative control is used to ensure that samples are not contaminated.

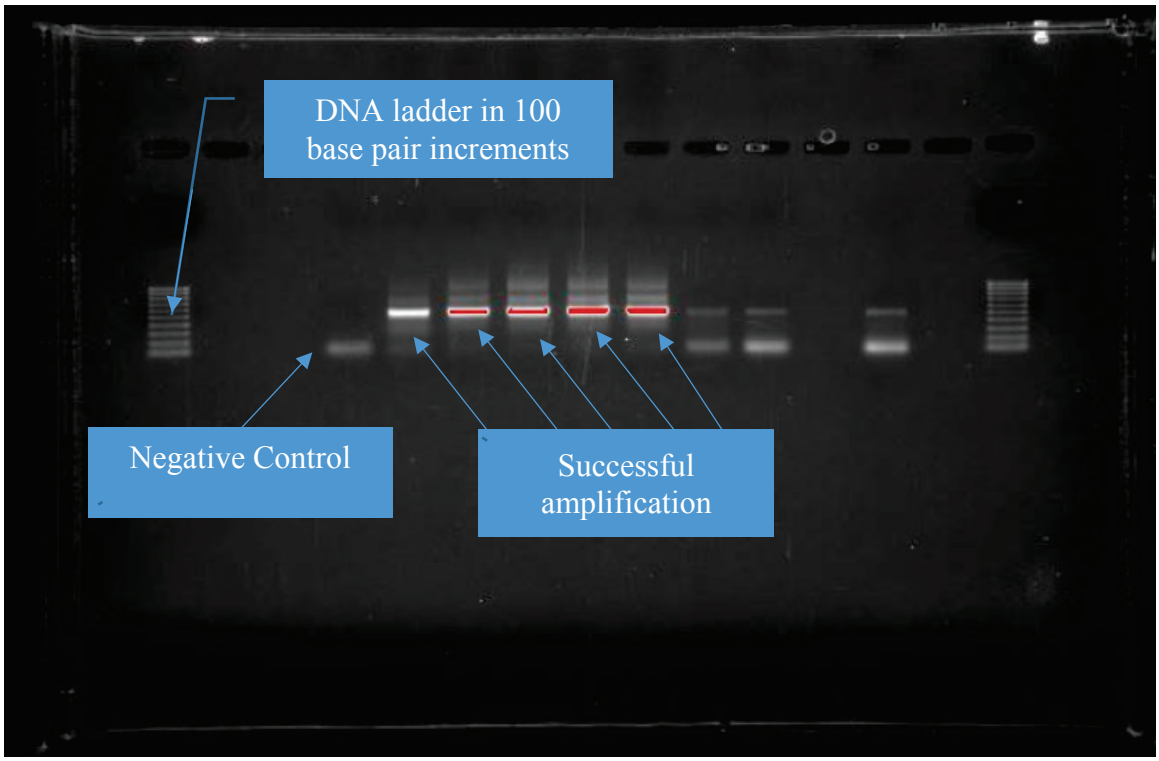

Figure G-2. This represents what a successful 341F/785R amplification looks like. Successful amplification would fall in the range of 400-500 based on the DNA ladder in lane 1. If bands are not reproduced in this range then PCR was unsuccessful. A negative control is used to ensure that samples are not contaminated.

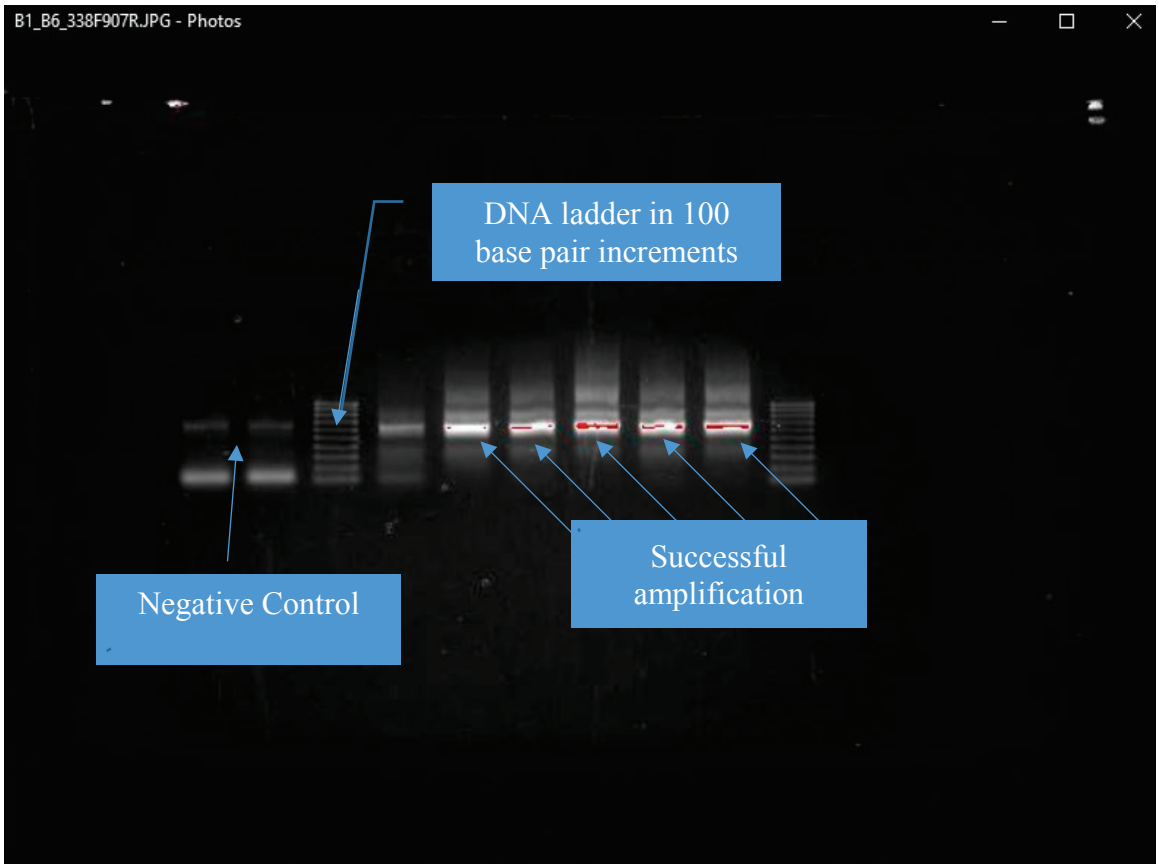

Figure G-3. This represents what a successful 338F/907R amplification looks like. Successful amplification would fall in the range of 500-600 based on the DNA ladder in lane 1. If bands are not reproduced in this range then PCR was unsuccessful. A negative control is used to ensure that samples are not contaminated.

Appendix H

Final CUGI Concentrations

Table H-1. This table displays the final concentrations of DNA after PCR amplification. These concentrations will be sent to CUGI for sequencing.

Bacteria			Bacteria			Archaeal		
338F 907R			341F 785R			1349F 1571R		
HB1	32.8	ng/µL	HB1	36	ng/µL	HB1	28	ng/µL
HB2	34.8	ng/µL	HB2	30	ng/µL	HB2	33.6	ng/µL
HB3	33	ng/µL	HB3	39.1	ng/µL	HB3	30.7	ng/µL
HB4	31.8	ng/µL	HB4	35.4	ng/µL	HB4	29.7	ng/µL
BB1	29.7	ng/µL	BB1	30.9	ng/µL	BB1	30.8	ng/µL
BB2	39.7	ng/µL	BB2	45.4	ng/µL	BB2	31.1	ng/µL
BB3	29.4	ng/µL	BB3	31.4	ng/µL	BB3	33.7	ng/µL
BB4	43.9	ng/µL	BB4	35.1	ng/µL	BB4	28.8	ng/µL
BB5	36.4	ng/µL	BB5	32.3	ng/µL	BB5	31.3	ng/µL
BB6	34.1	ng/µL	BB6	31.9	ng/µL	BB6	32.2	ng/µL
1.1	29.3	ng/µL	1.1	34.6	ng/µL	1.1	30.1	ng/µL
1.2	35.8	ng/µL	1.2	35.5	ng/µL	1.2	29.2	ng/µL
1.3	34.3	ng/µL	1.3	49.8	ng/µL	1.3	34.6	ng/µL
1.4	33.7	ng/µL	1.4	47.3	ng/µL	1.4	31	ng/µL
2.1	29.7	ng/µL	2.1	42	ng/µL	2.1	32.2	ng/µL
2.2	44.8	ng/µL	2.2	43	ng/µL	2.2	30.1	ng/µL
2.3	35.1	ng/µL	2.3	33	ng/µL	2.3	31.3	ng/µL
2.4	30.5	ng/µL	2.4	43.6	ng/µL	2.4	37.4	ng/µL
3.1	31.3	ng/µL	3.1	27.8	ng/µL	3.1	29.4	ng/µL
3.2	294	ng/µL	3.2	30.4	ng/µL	3.2	30.5	ng/µL
3.3	28.8	ng/µL	3.3	50	ng/µL	3.3	57	ng/µL
3.4	27.5	ng/µL	3.4	59	ng/µL	3.4	32.5	ng/µL
4.1	28.6	ng/µL	4.1	37.4	ng/µL	4.1	37.9	ng/µL
4.2	33.4	ng/µL	4.2	35.1	ng/µL	4.2	28.9	ng/µL
4.3	45.1	ng/µL	4.3	51	ng/µL	4.3	29.3	ng/µL
4.4	35.7	ng/µL	4.4	30.7	ng/µL	4.4	27.8	ng/µL

Appendix I

Batch Study Results

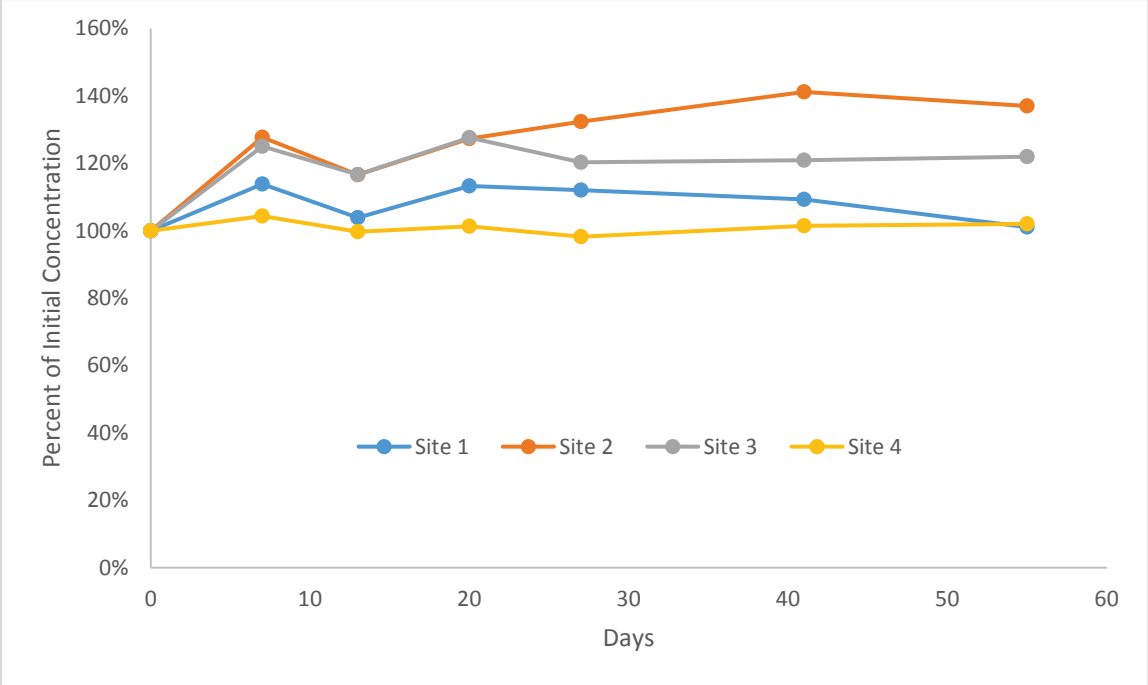

Figure I-1. This graph represents the kill control amended with MTBE for the four Houston sites. Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

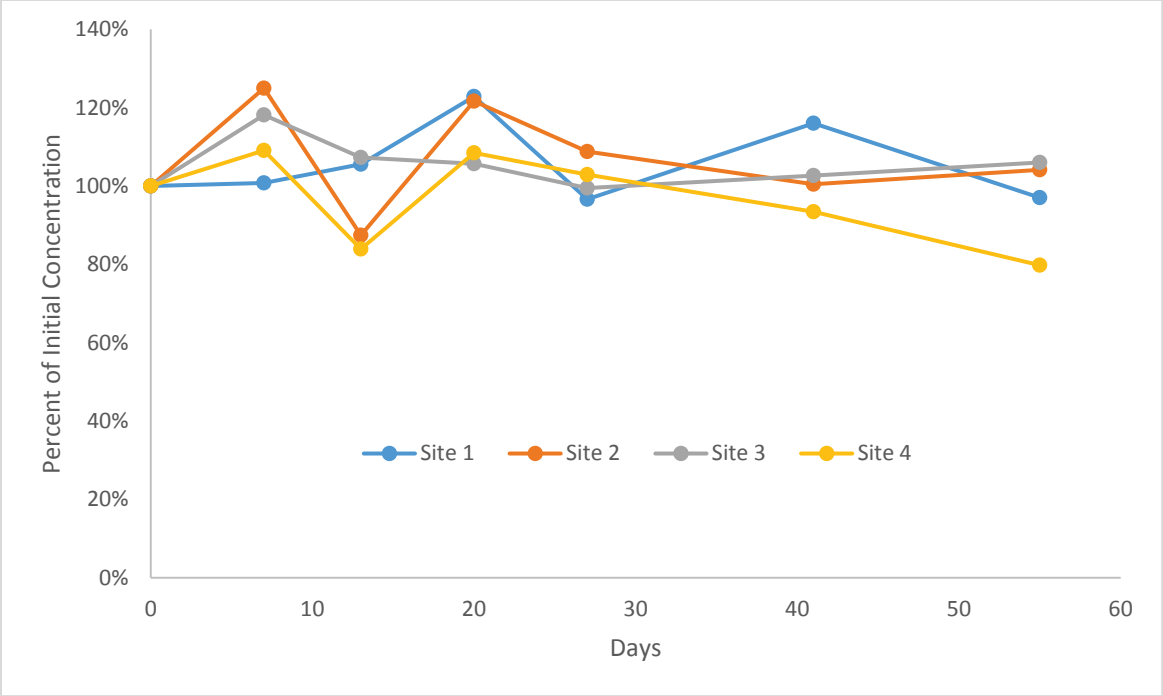

Figure I-2. This graph represents the MTBE without any other electron donor addition for the four Houston sites. Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

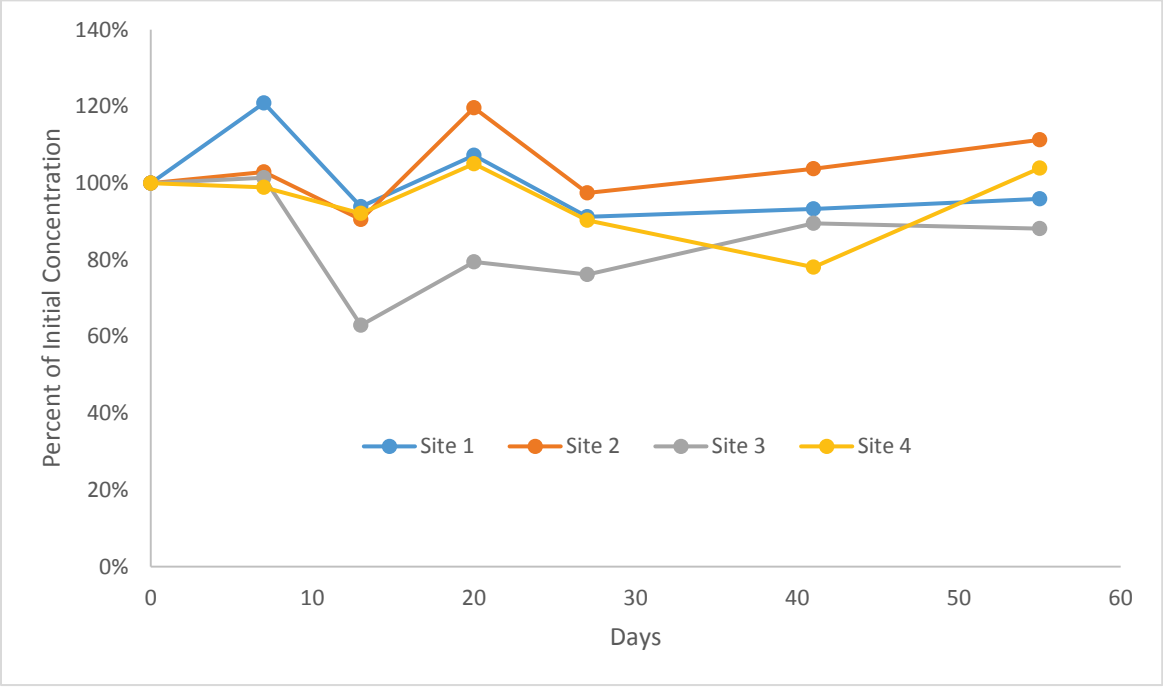

Figure I-3. This graph represents the four Houston sites amended with MTBE and fumarate. Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

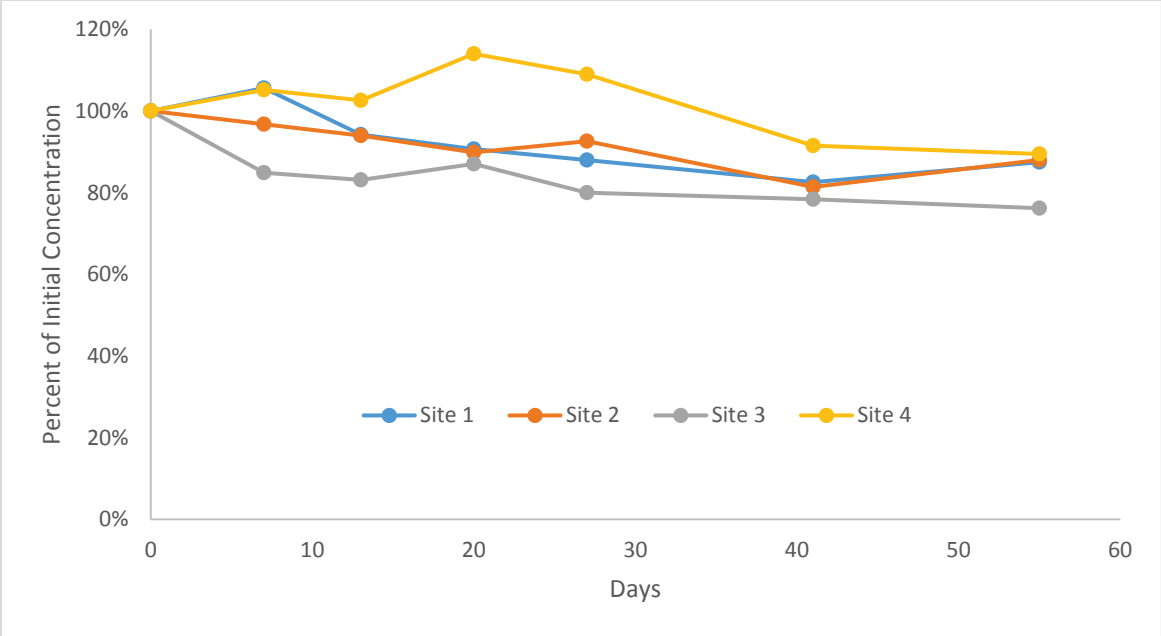

Figure I-4. This graph represents the four Houston sites amended with MTBE and Fe(III). Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

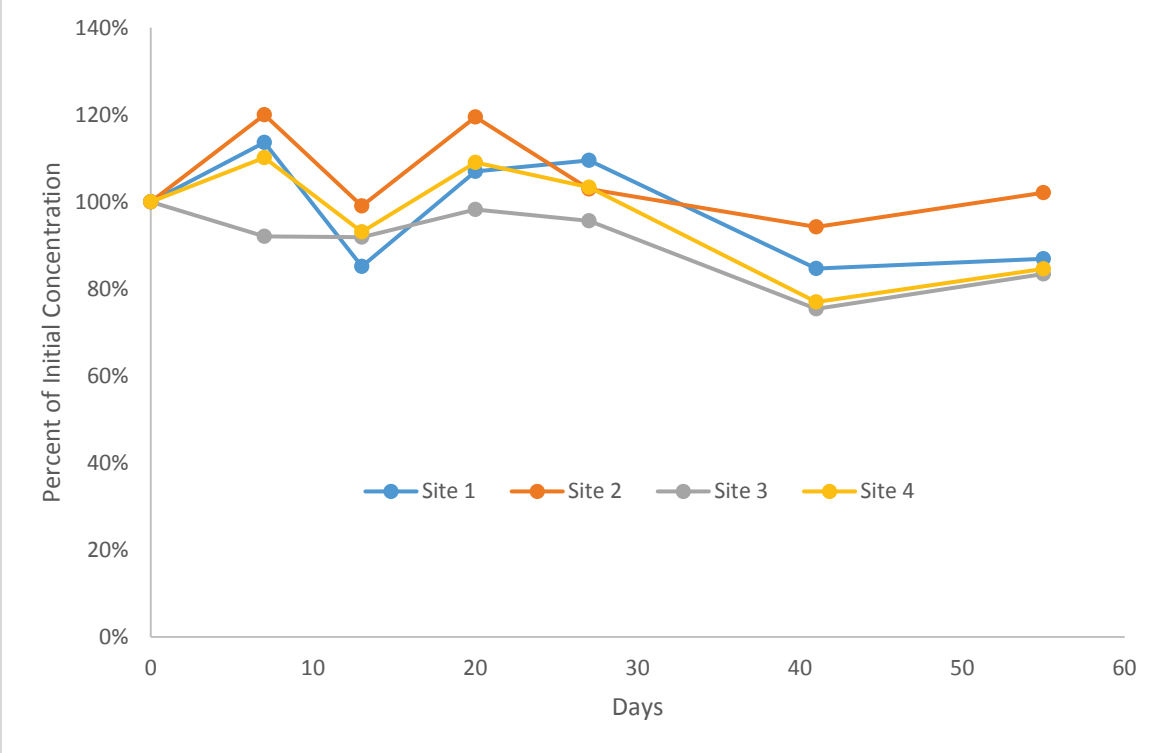

Figure I-5. This graph represents the four Houston sites amended with MTBE, Fe(III), and AQDS. Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

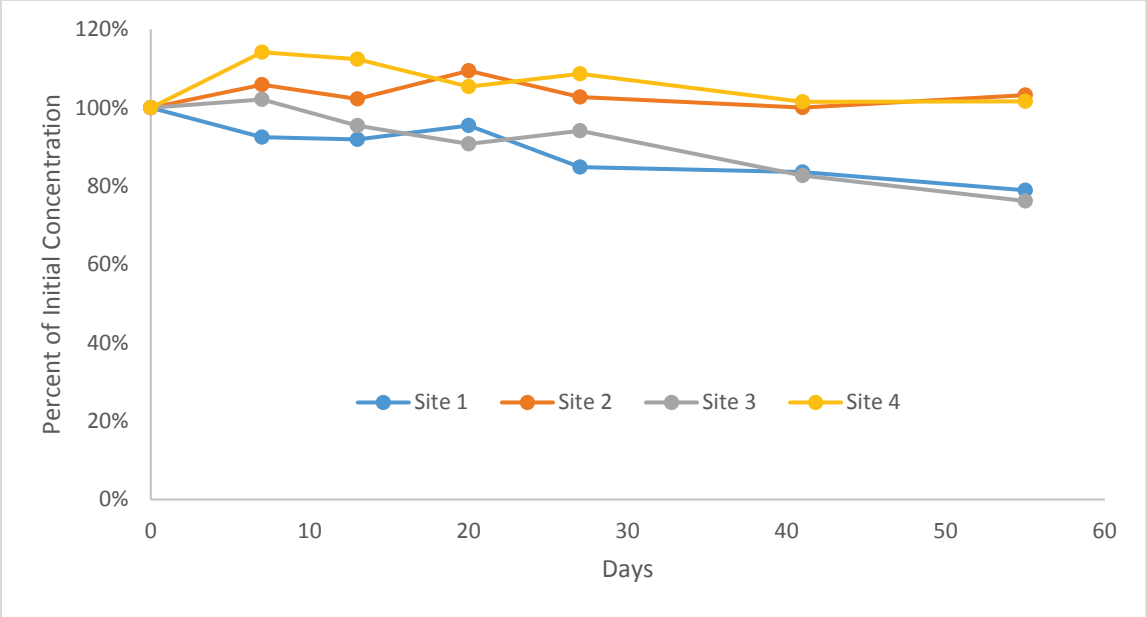

Figure I-6. This graph represents the four Houston sites amended with MTBE and nitrate. Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

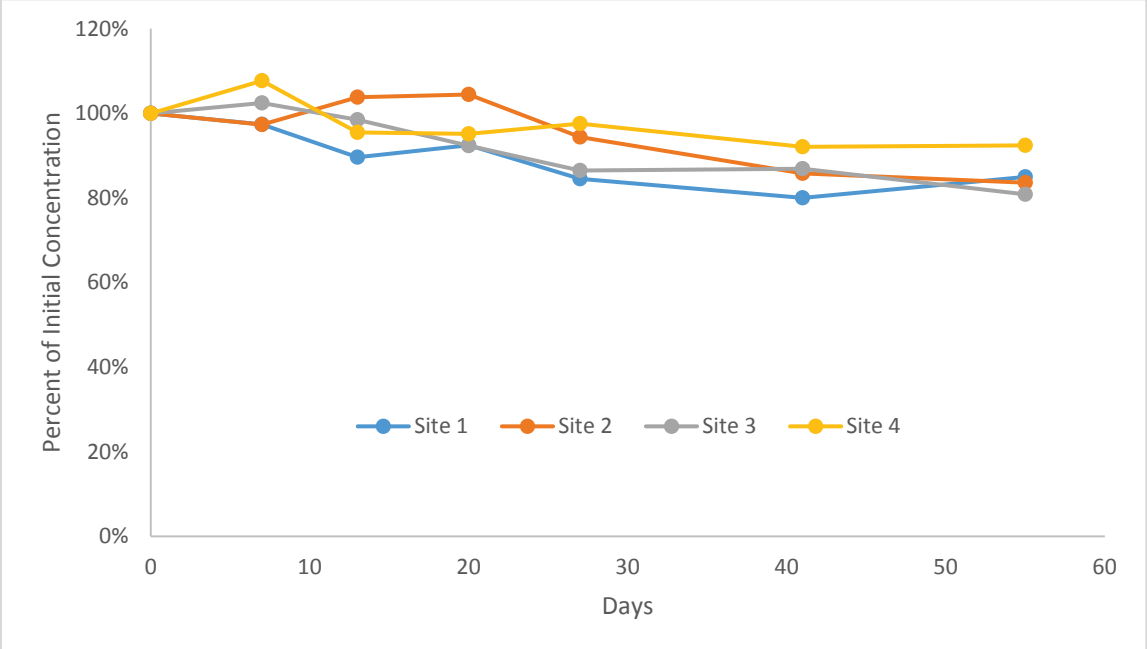

Figure I-7. This graph represents the four Houston sites amended with MTBE and sulfate. Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

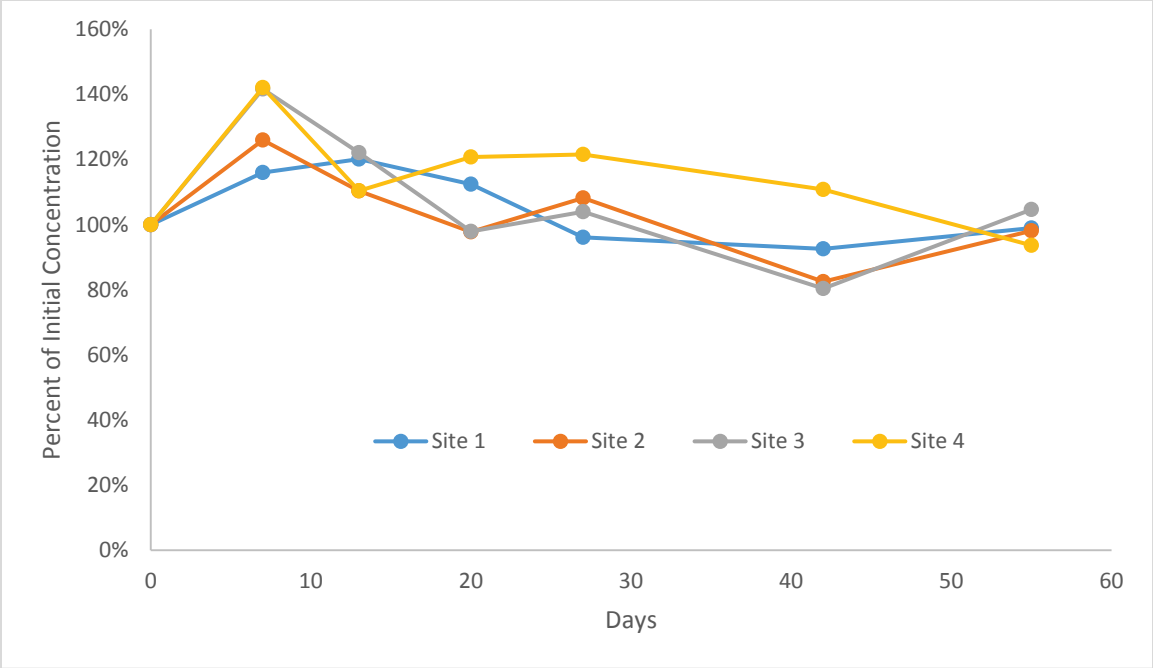

Figure I-8. This graph represents the kill control amended with TBA for the four Houston sites. Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

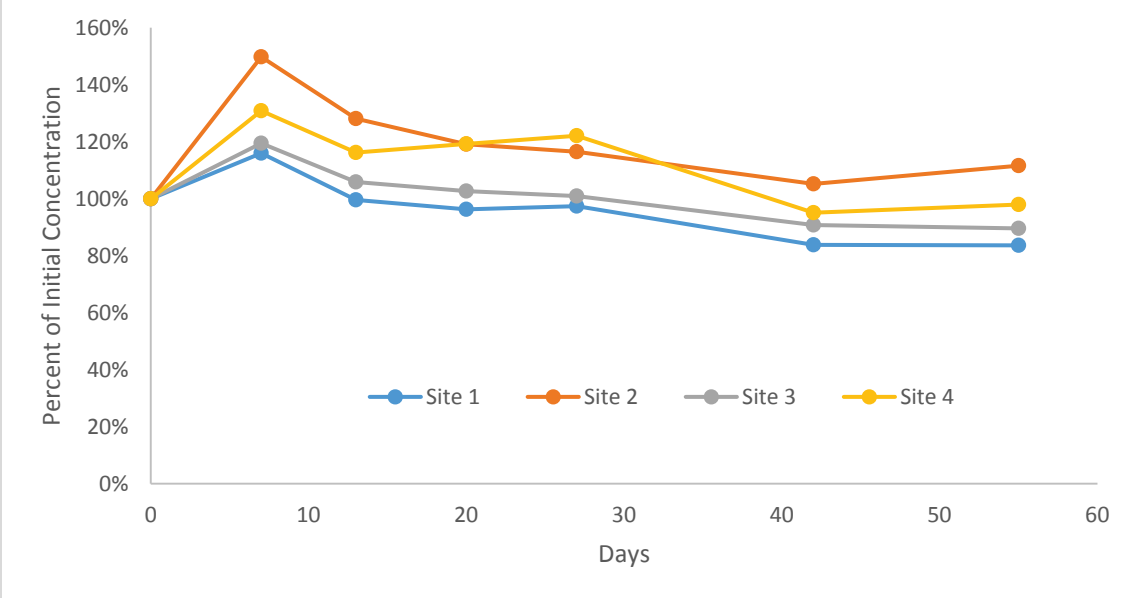

Figure I-9. This graph represents the TBA without any other electron donor addition for the four Houston sites. Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

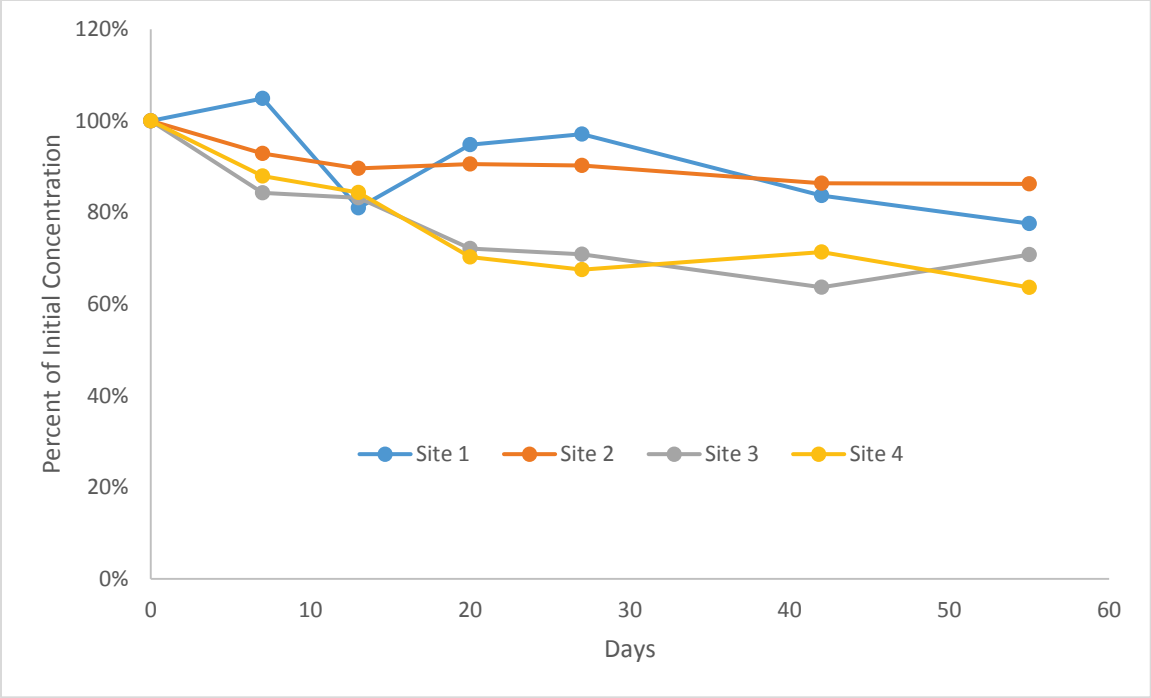

Figure I-10. This graph represents the four Houston sites amended with TBA and fumarate. Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

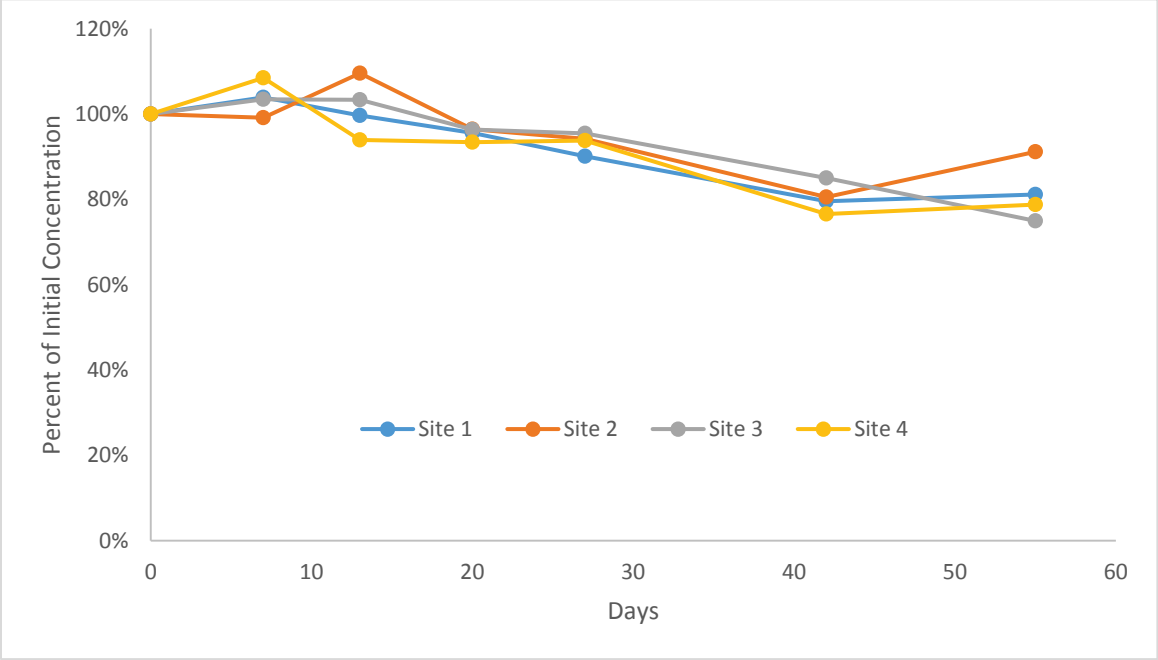

Figure I-11. This graph represents the four Houston sites amended with TBA and Fe(III). Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

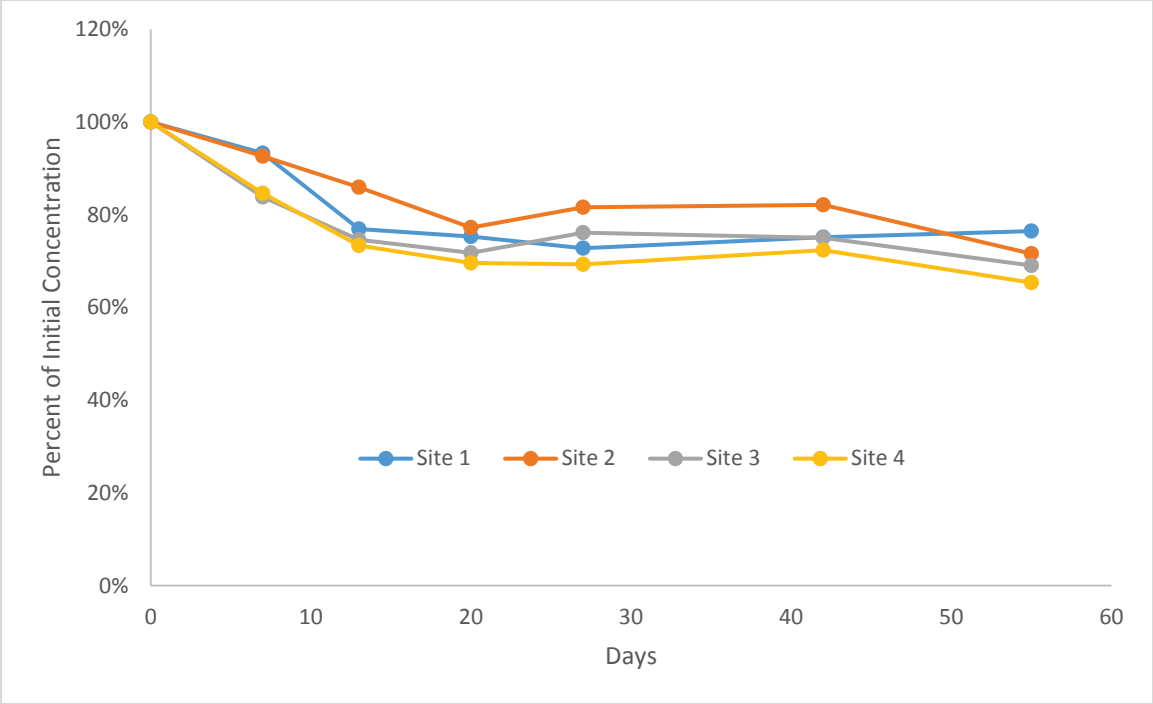

Figure I-12. This graph represents the four Houston sites amended with TBA, Fe(III), and AQDS. Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

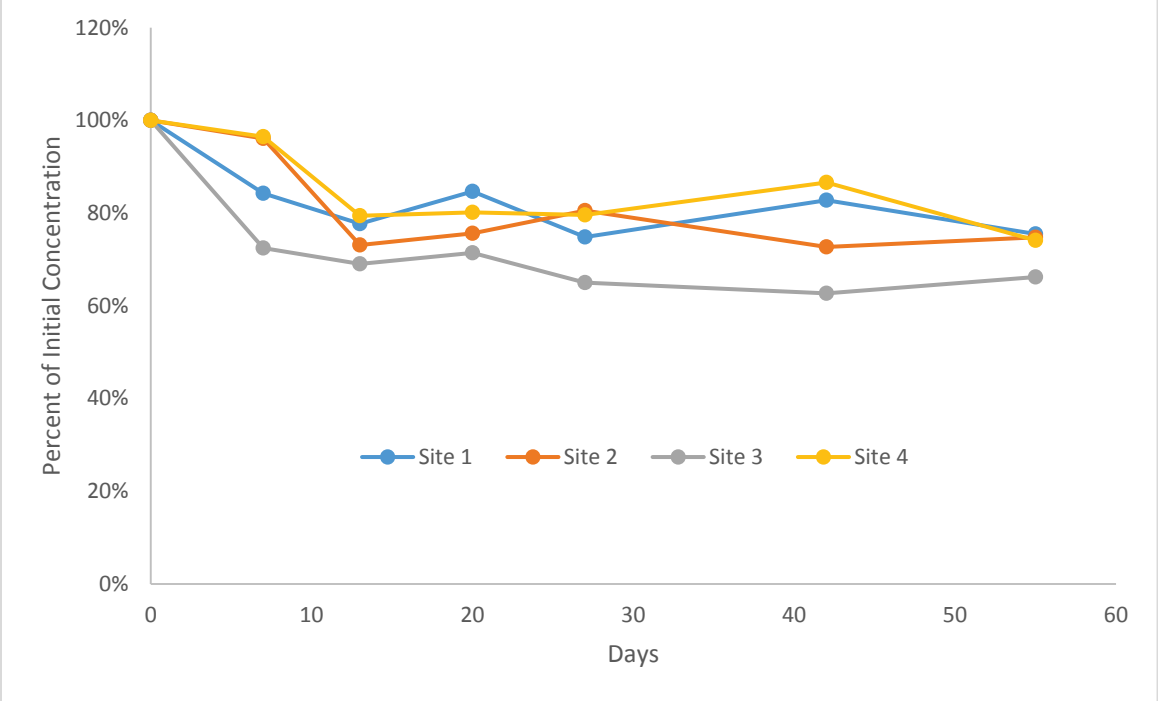

Figure I-13. This graph represents the four Houston sites amended with TBA and nitrate. Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

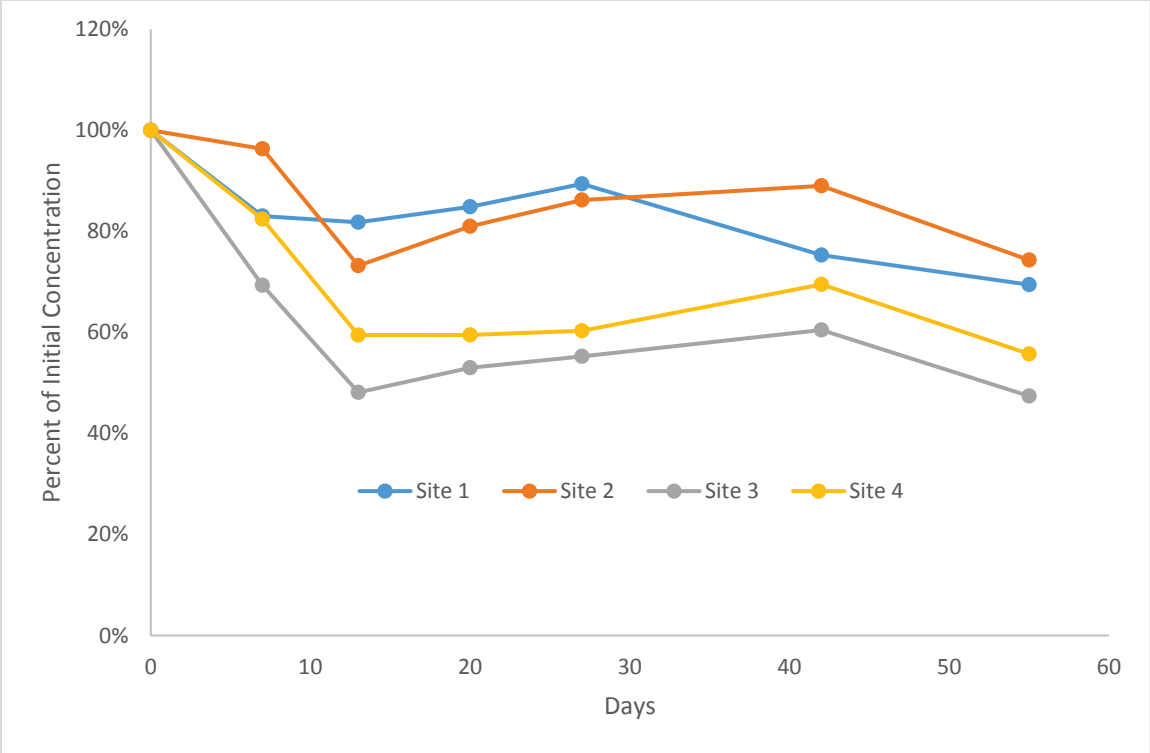

Figure I-14. This graph represents the four Houston sites amended with TBA and sulfate. Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

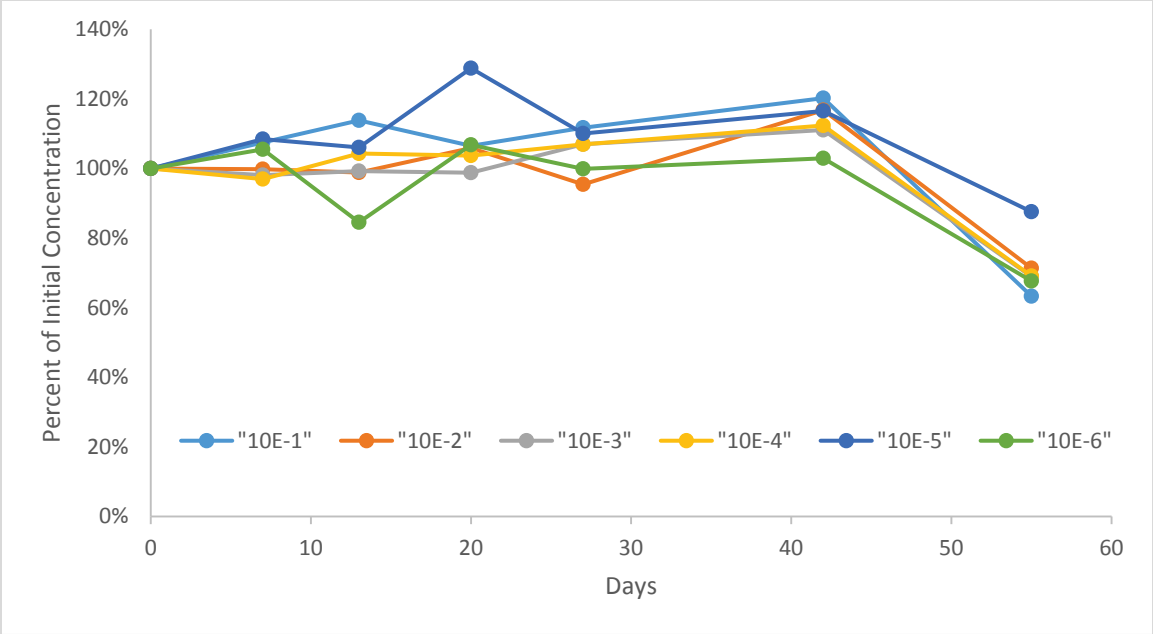

Figure I-15. Most Probable Number (MPN) dilution series amended with MTBE, starting with a Houston site four MTBE amended bottle. Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

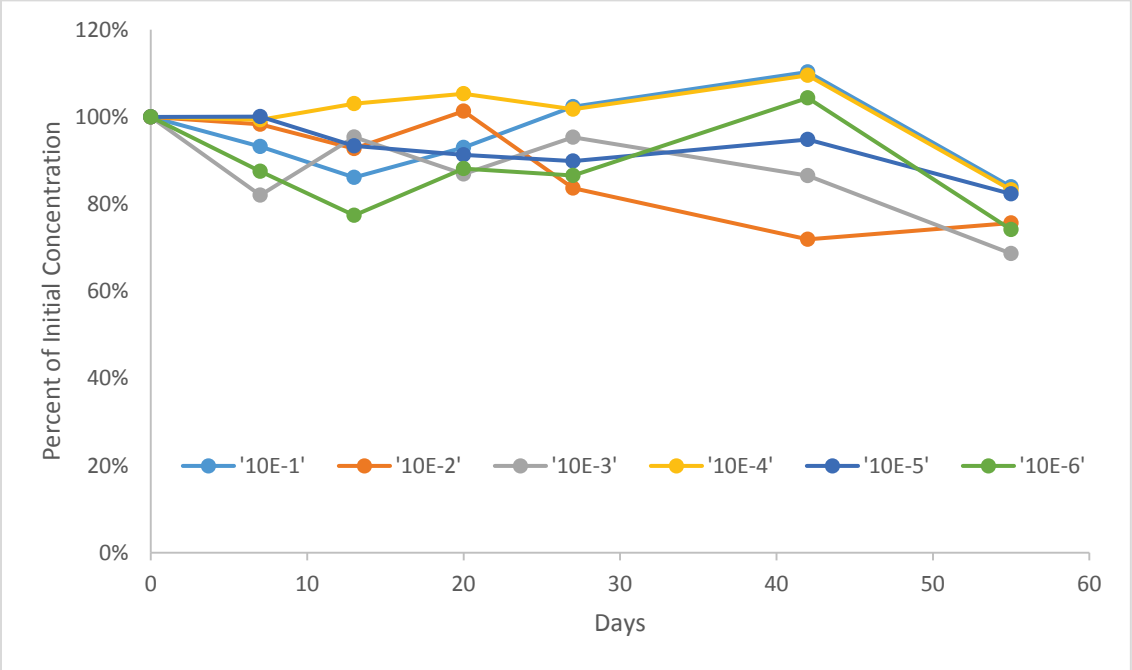

Figure I-16. Most Probable Number (MPN) dilution series amended with MTBE, starting with a Battelle site three MTBE amended bottle. Percent of initial concentration represents the remaining concentration based on a starting concentration of 1 mM MTBE.

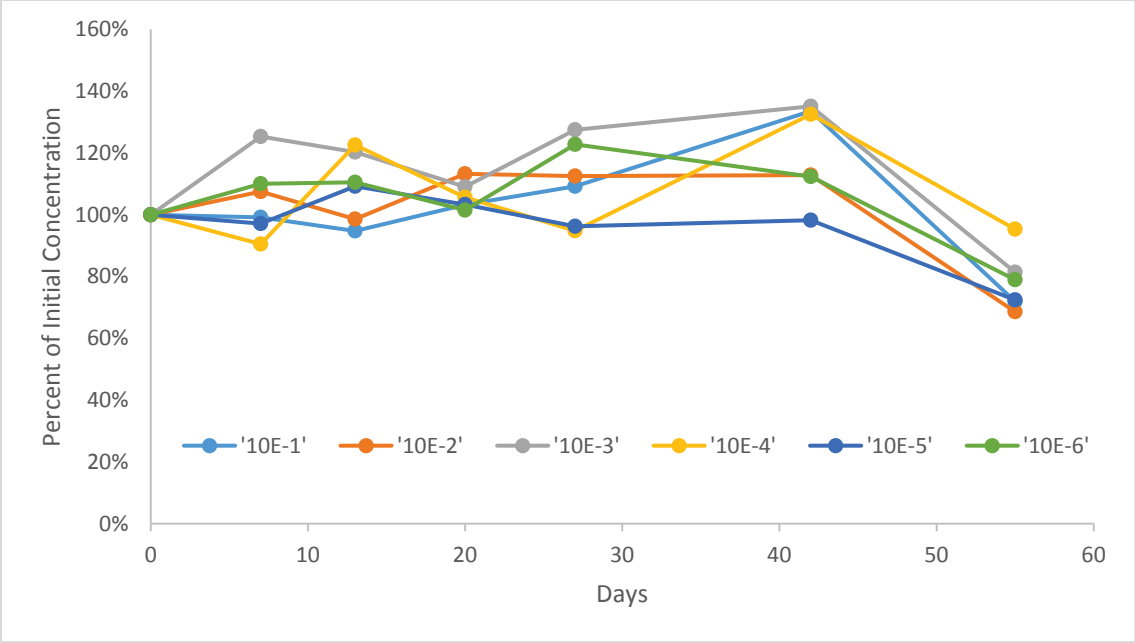

Figure I-17. Most Probable Number (MPN) dilution series amended with TBA, starting with a Houston site three TBA amended bottle. Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

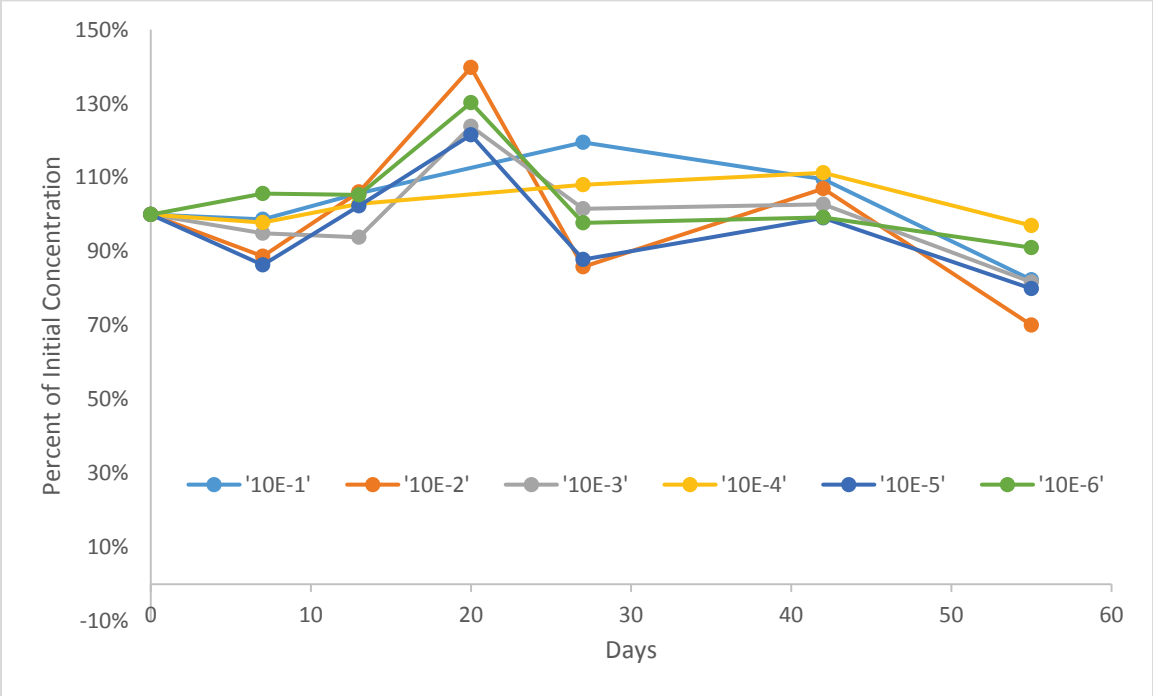

Figure I-18. Most Probable Number (MPN) dilution series amended with TBA, starting with a Battelle site six TBA amended bottle. Percent of initial concentration represents the remaining concentration based on a starting concentration of 5 mM TBA.

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